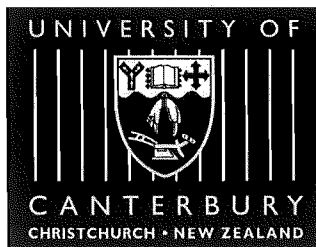


**Plant Tissue Culture Investigations on *Capsicum annuum* L. :  
Somatic Embryogenesis, *in vitro* Flowering,  
Fruiting and Seed Formation**

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A thesis  
submitted in fulfillment  
of the requirements for the Degree  
of  
Doctor of Philosophy in Plant Biotechnology  
in the  
University of Canterbury  
by  
**Bodhipadma, Kitti**

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For my beloved wife, Supanya, who has always given me her happiness,  
shared my suffering, and cheered me up.

# Abstract

Four cultivars of *Capsicum annuum* L., namely Sweet Banana, California Wonder, Yolo Wonder and Ace were used in different experiments initially to examine more closely some of the critical factors influencing somatic embryogenesis in zygotic embryo explants. Using the protocol of Buyukalaca and Mavituna (1996) only callus formation without embryogenesis was observed in the mature zygotic embryo explants of the four cultivars. With some modifications to the protocols of Harini and Lakshmi Sita (1993) and Binzel et al. (1996), improved somatic embryogenesis in immature zygotic embryo explants of the cultivars used in the present study was achieved. When the immature zygotic embryo explants were placed on induction medium longer than 2 weeks, the percentages of plantlet conversion were significantly reduced from 75% and 65% to 40% and 28% in cvs. Sweet Banana and California Wonder, respectively. Somatic embryogenesis of cv. Yolo Wonder was not observed if sucrose was not added to the induction medium or if it was supplemented either with 10% glucose or fructose. Maltose could replace sucrose as far as somatic embryo induction and eventual plantlet conversion in the cv. Yolo Wonder is concerned. For all 4 cultivars, somatic embryos were initiated from immature zygotic explants on medium with or without coconut water, in the light or dark condition.

Somatic embryo structures of *C. annuum* L. cv. Sweet Banana were easily observed under a stereo-microscope after 14 days of culture on the induction medium. These structures turned green 5-7 days after the explants were transferred to the germination or conversion medium, and then they germinated into plantlets about 3 days later. The origin and developmental sequence of the somatic embryos were investigated using the resin infiltration and embedding technique. Periclinal cell division firstly began underneath the protoderm of the cotyledons of the explant after 3 days of culture on somatic embryo induction medium. The number of cells obviously increased after 5-7 days of culture as a result of both periclinal and anticlinal cell divisions. After 14 days, the globular structures were clearly visible on the surface of the cotyledons of the immature zygotic embryo explant. Upon further culture, other well known somatic embryo developmental stages, including heart-shaped, torpedo and cotyledonary stages were observed.

The germinated somatic embryos of *C. annuum* L. cv. Sweet Banana grew well on the agar-gelled plantlet development medium comprising the basal salts and vitamin

mixture of Murashige and Skoog (1962) supplemented with 1 mg/l NAA and no sucrose. Upon further culture, the somatic embryo-derived plantlets were found to be capable of flowering *in vitro* in small tissue culture containers. The *in vitro* flowers were compared with those produced by the mature plants grown in the glasshouse. It was found that there were no obvious differences in the outer parts (calyx colour, corolla number, corolla colour and corolla length) of flowers from both sources. The inner parts of both *in vitro* and *in vivo* flowers were slightly different as far as stamen number, anther length, filament length, carpel length and style length are concerned. However, anther colour, filament colour, carpel number, carpel colour, style colour, and stigma colour were the same. Pollen from both *in vitro* and *in vivo* flowers also appeared to be the same when observed under a scanning electron microscope. In contrast, pollen physiology of *in vitro* and *in vivo* flowers in relation to both viability and germination tests were slightly different (ANOVA,  $P < 0.05$ ). The percentages of viability and germination of pollen from *in vitro* flowers were about 94% and 25%, respectively, while those of pollen from *in vivo* flowers were about 96% and 34%, respectively. *In vitro* floral buds were first formed on plantlets that grew on the plantlet development medium in a growth room at 22 °C under continuous illumination provided by white fluorescent lamps. However, the floral buds rarely developed further into mature flowers as they abscised soon after their formation. This problem was overcome using the vented autoclavable plant tissue culture containers (Phytakon<sup>TM</sup>, Sigma Chemical Co.). *In vitro* fruit formation and ripening was observed when liquid half strength MS basal medium supplemented with 5 µg/ml Ag<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, 1 mg/l NAA and 3% sucrose was added to the surface of the plantlet development medium. Further improvement to this *in vitro* fruiting system was achieved with the aid of hand pollination.

Clonal propagation starting with both nodal and excised shoot tip explants from the plantlets of *C. annuum* L. cv. Sweet Banana regenerated via somatic embryogenesis was investigated. It was found that sucrose in MS basal medium free of plant growth regulators is an important factor for plantlet regeneration from the explants. Furthermore, the plantlets were capable of flowering, fruit setting, fruit ripening and seed setting *in vitro*. The harvested seeds germinated and developed into normal-looking seedlings. This is the first report on the completion under *in vitro* conditions of the whole developmental cycle starting from somatic embryogenesis and resulting in the formation of germinable seeds of *C. annuum* L.



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# Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
ABA	abscisic acid
AgNO <sub>3</sub>	silver nitrate
AGP	arabinogalactan protein
Ag <sub>2</sub> S <sub>2</sub> O <sub>3</sub>	silver thiosulfate (STS)
BA	6-benzyladenine
dH <sub>2</sub> O	distilled water
EC	embryogenic clumps
GA <sub>3</sub>	gibberellic acid
βGluY	Yariv's reagent
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
MS	Murashige and Skoog
NAA	α-naphthaleneacetic acid
PAA	phenyl acetic acid
PEM	proembryogenic mass
SE	somatic embryogenesis
SEM	scanning electron microscope
TEM	transmission electron microscope
TDZ	thidiazuron
TIBA	2,3,5-triiodobenzoic acid

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# Chapter 1

## Introduction

### 1.1 Tissue culture of *Capsicum annuum* L.

#### 1.1.1 A definition of plant tissue culture

Generally, plant tissue culture is defined as the process in which protoplasts, cells, tissues and organs are isolated from the plant body and grown or maintained aseptically in solid or liquid medium (Dodds and Roberts, 1987; Arditti and Ernst, 1993; Phillips and Gamborg, 1995; Ignacimuthu, 1997). Plant tissue culture has become one of the core technologies in plant biotechnology (Brown and Thorpe, 1995). The application of most, if not all, plant transformation or genetic modification techniques, is either dependent on or greatly assisted with reliable and efficient *in vitro* plant regeneration technology.

#### 1.1.2 *Capsicum* (peppers)

*Capsicum* peppers are native plants of the New World tropics. They belong to the family Solanaceae which also includes potato, tomato and tobacco. Five different species, *Capsicum annuum*, *C. frutescens*, *C. chinense*, *C. baccatum* and *C. pubescens*, are regarded as domesticated peppers. There is evidence that humans began to use them before the beginning of agriculture (Pickersgill, 1969; IBPGR, 1983; Langer and Hill, 1991).

The domesticated peppers are used as spices in various forms or vegetables, for example, dried powders of different colours, paprika, Tabasco, pungent chilli peppers, pimentos, red and cayenne peppers and sweet or bell peppers. Besides their use as food and condiment, peppers have some application in medicine and some have ornamental values (Purseglove et al., 1981; IBPGR, 1983; Morrison et al., 1986).

The mild or sweet peppers (*Capsicum annuum* L.) are indigenous to Central America, and were taken by Christopher Columbus to Europe. The fruits have a unique flavour and contain a high level of vitamins C and A. They are consumed raw, pickled or cooked and can hardly be called a spice. In New Zealand, bell peppers (*C. annuum* L.) in brilliant red, yellow, orange, and sometimes purple/black have become popular vegetables in the last ten years (Hartmann et al., 1981; Purseglove et al., 1981; Toussaint-Samat, 1993; McGeorge, 1997).

*C. annuum* L. is an erect annual plant reaching up to 150 cm in height, while the other species are generally perennial shrubs. An adult *C. annuum* L. plant has a single stem with 8-15 leaves before the emergence of the first terminal flower. When the first floral bud develops, branching at the apex into two or more shoots starts. Each shoot has one or two leaves, terminates in a flower, and then divides into two second-order branches. The two lateral branches form a dichasium, and the terminal bud develops into a floral apex. One of the dichotomic branches is sometimes suppressed, especially in the third- and higher-order branches, so that the branch system evolves towards a sympodium. The root system consists of a main taproot and extensively branched lateral roots system. Leaves are very variable in shape but remain simple. The flower is monoecious and solitary. The calyx is bell-shaped with five teeth, and the corolla is wheel- to bell-shaped with five to six parts, which are white or green. There are five or six stamens inserted near the base of the corolla. The period of anthesis is relatively short. Self-pollination is common but a high rate of cross-pollination also occurs. The fruits are produced individually at the node. The range of their shapes and colours far exceeds that of other *Capsicum* species. Seed set and seed production within the fruits are improved by pollination with a large amount of pollen from many plants (Tanksley, 1984; Ryłski, 1985; Ryłski, 1986; Langer and Hill, 1991).

*C. annuum* L. plants do not tolerate frost. Therefore, the crop can only be grown successfully in regions with more than three frost-free months in a year. Flowering begins one to two months after seed germination and one month later many fruits are ready for harvest at the green-mature stage. Fruit yields are higher when temperature ranges from 20 to 30 °C while extremely high temperatures result in poor fruiting (Hartmann et al., 1981; Purseglove et al., 1981; Langer and Hill, 1991).

### 1.1.3 *Capsicum annuum* L. tissue culture

Many reviews (Fari, 1986; Morrison et al., 1986; Vagera, 1990; Fari and Andrasfalvy, 1994; Ezura, 1997; Steinitz et al., 1999) have concluded that research on *Capsicum* tissue culture is less advanced compared to that on other species in the same family, especially tobacco, tomato and eggplant. For *C. annuum* L., plant regeneration has been achieved using many types of explants. These include protoplasts (Saxena et al., 1981; Diaz et al., 1988; De Donato et al., 1989; Prakash et al., 1997), half-seeds (Ezura et al., 1993; Binzel et al., 1996a; Choi et al., 1996), mature zygotic embryos (Agrawal and Chandra, 1983; Christopher et al., 1986), hypocotyls (Fari and Cazko, 1981; Ochoa-Alejo and Garcia-Bautista, 1990; Ochoa-Alejo and Ireta-Moreno, 1990; Christopher et al., 1991; Ramirez-Malagon and Ochoa-Alejo, 1996), cotyledons (Sripichitt et al., 1987; Subhash and Prolaram, 1987; Sripichitt et al., 1988; Fras and Nowak, 1995; Husain et al., 1999), leaves (Kintzios et al., 1996), and shoot tips (Fari and Csillery, 1983; Agrawal et al., 1988; Christopher and Rajam, 1994). Among these, hypocotyls and cotyledons appear to be the most popular explants (Gunay and Rao, 1978; De Donato et al., 1984; Phillips and Hubstenberger, 1985; Agrawal et al., 1989; Arroyo and Revilla, 1991; Morone-Fortunato and Tudisco, 1991; Rogozinska and Tobolewska, 1992; Ebida and Hu, 1993; Shao and Caponetti, 1993; Christopher and Rajam, 1996; Berljak, 1999).

*In vitro* production of haploid *C. annuum* L. plants from anther explants could occur both via organogenesis and embryogenesis. *C. annuum* L. anther culture was first successfully established using microspores at the uninucleate stage (Wang et al., 1973). The production of callus and embryoids from *Capsicum* anthers was found to be dependent on plant growth regulators. Most embryoids became degenerated during development (Kuo et al., 1973) and merely up to 0.001% survived as viable plantlets (George and Narayanaswamy, 1973). However, some experiments found only callus formation from microspores and anthers (Novak, 1974; Munyon et al., 1989). This may be related to the use of different cultivars in the various studies (Yoon et al., 1991).

The optimum developmental state of pollen to form a high frequency of haploid embryoids at 35 °C was late uninucleate or approximately during the first microspore mitosis (Harn et al., 1975; Sibi et al., 1979; Morrison et al., 1986; Vagera, 1990). Recently, a satisfactory number of haploid plantlets from anther culture of *C. annuum* L. was obtained in a single step procedure involving the use of maltose, liquid-solid medium

and the high CO<sub>2</sub> (Dolcet-Sanjuan et al., 1997). Furthermore, a cytological investigation showed that the formation of embryoids from anther culture of *C. annuum* L. began only when the uninucleated microspore divided into two symmetrical vegetative nuclei (Barcaccia et al., 1999). Thus far, isolated microspore culture of *C. annuum* L. has been unsuccessful (Regner, 1996), and needs more research.

## **1.2 Somatic embryogenesis**

### **1.2.1 Somatic embryo**

Steward et al. (1958) and Reinert (1958, 1959) both proved Haberlandt's idea of "totipotency" of plant cell for the first time by demonstrating somatic embryogenesis in carrot cell suspension cultures and carrot callus grown on semisolid medium, respectively (Krikorian and Berquam, 1969; Ammirato, 1984). Somatic embryogenesis is a developmental process by which non-zygotic embryos, named somatic embryos, are initiated from a single or group of sporophytic cells without reductive cell divisions and fertilisation of sex cells that lead to the formation of zygotic embryos. Somatic embryogenesis is different from organogenesis because a somatic embryo is a bipolar structure capable of developing into a plantlet without separate shoot and root initiation stages. Somatic embryos are capable of germinating and developing into the whole plants. However, a somatic embryo develops in the absence of the seed coat and endosperm that normally provide protection and nutrients for the zygotic embryo in a developing seed (Kohlenbach, 1978; Ammirato, 1983; Bajaj, 1995; Phillips et al., 1995; Schumann et al., 1995; McKersie and Brown, 1996; Ignacimuthu, 1997).

### **1.2.2 Practical importance of somatic embryogenesis**

With the understanding that somatic embryogenesis is not restricted to a few species or genera has come the confident expectation that it is an indispensable requirement for the application of many tools in plant biotechnology (Tisserat et al., 1979; Ammirato, 1983; Bajaj, 1995; McKersie and Brown, 1996). In particular, somatic embryogenesis (SE) may play an increasing role in plant biotechnological approaches to plant improvement in the



following ways (Levin et al., 1988; Komamine et al., 1992; Attree and Fowke, 1995; Bajaj, 1995; Gray, 1996; Merkle, 1997; Jain, 1999):

- (a) application of SE for cost effective, high-volume and large-scale clonal propagation of elite of breeding materials;
- (b) application of SE for rapid bulking up of valuable genotypes;
- (c) long-term storage by cryopreservation and establishment of germplasm banks using somatic embryos; and
- (d) genetic transformation and the production of transgenic plants starting with explants that can be subsequently induced to form somatic embryos.

### **1.2.3 Direct somatic embryogenesis**

Direct or adventitious somatic embryogenesis occurs directly in an explant without the formation of an intermediate callus phase. This suggests the presence of cells, which are pre-determined or programmed for embryonic development prior to explanting. These cells require only growth regulators or favourable conditions to allow activation of cell division and expression of the embryogenesis programme (Evans et al., 1981; Williams and Maheswaran, 1986).

Direct somatic embryogenesis commonly resulting in clumps of embryos arising directly from an explant. Immature zygotic embryos are most often used to induce direct somatic embryogenesis. This tissue is already embryogenic in nature and seemingly demands less nourishing than other somatic tissues for a somatic embryogenic response to occur (Finer, 1995).

### **1.2.4 Indirect somatic embryogenesis**

Indirect somatic embryogenesis involves a sequence of redetermination of differentiated cells, callus proliferation, and subsequent development of a determined state for embryogenesis. Somatic embryos originate from induced cells within the callus. Growth regulators are required not only for the re-entry of a differentiated cell into mitosis but also

for determination of the embryogenic state (Sharp, 1980; Evans et al., 1981; Williams and Maheswaran, 1986).

Plant regeneration by indirect somatic embryogenesis was originally demonstrated using carrot cell suspension culture (Steward et al., 1958; Reinert, 1959). In this process, many well-defined intermediate embryo development stages were evident, including the globular, heart, torpedo, cotyledonary, and mature stages (Phillips et al., 1995). Indirect somatic embryogenesis is preferable than the direct process as the former is amenable to scale-up mass propagation of plants and efficient gene transfer (Merkle, 1997).

### 1.2.5 Sources of somatic embryos

Somatic embryos can be obtained from many sources of explants (Kohlenbach, 1978; Bhojwani and Razdan, 1983). In this thesis, somatic embryogenesis of a member of the Solanaceae family is of interest and the major relevant studies are summarised in Table 1.1.

**Table 1.1** List of plant species and the different explants that are capable of forming somatic embryos in the family Solanaceae. Plant classification is based on D'Arcy (1979) and Judd et al. (1999).

Plant	Explant	Authorities
<i>Atropa belladonna</i> L.	Anther	see (Tisserat et al., 1979)
<i>A. belladonna</i> L.	Callus	see (Tisserat et al., 1979)
<i>A. belladonna</i> L.	Protoplast	(Gosch et al., 1975; Lorz and Potrykus, 1979)
<i>A. belladonna</i> L.	Root	(Thomas and Street, 1970; Konar et al., 1972; Thomas and Street, 1972)

**Table 1.1** (continued)

<b>Plant</b>	<b>Explant</b>	<b>Authorities</b>
<i>Capsicum annuum</i> L.	Anther	see 1.1.3 <i>Capsicum annuum</i> L. tissue culture
<i>C. annuum</i> L.	Immature embryo	(Harini and Lakshmi Sita, 1993; Binzel et al., 1996b; Jo et al., 1996)
<i>C. annuum</i> L.	Leaf	(Kintzios et al., 2000; Kintzios et al., 2001)
<i>C. annuum</i> L.	Embryo	(Buyukalaca and Mavituna, 1996; Mavituna and Buyukalaca, 1996)
<i>Datura innoxia</i> Mill.	Anther and microspore	see (Tisserat et al., 1979) and (Sharma and Kothari, 1990)
<i>D. innoxia</i> Mill.	Immature embryo	(Ducrocq et al., 1994)
<i>D. metel</i> L.	Anther	see (Tisserat et al., 1979)
<i>D. meteloides</i> Dun.	Anther	see (Tisserat et al., 1979)
<i>D. stramonium</i> L.	Anther	see (Tisserat et al., 1979)
<i>D. wrightii</i> Regel	Anther	see (Tisserat et al., 1979)
<i>Hyoscyamus albus</i> L.	Anther	(Raghavan, 1990)
<i>H. muticus</i> L.	Protoplast	(Wernicke et al., 1979)
<i>H. niger</i> L.	Anther	(Raghavan, 1990)
<i>H. niger</i> L.	Embryo	(Tu et al., 1996)
<i>H. pusillus</i> L.	Anther	(Raghavan, 1990)

**Table 1.1** (continued)

<b>Plant</b>	<b>Explant</b>	<b>Authorities</b>
<i>Lycium barbarum</i> L.	Anther	(Cao et al., 1999a; Cao et al., 1999b)
<i>L. barbarum</i> L.	Leaf	(Kairong et al., 1999a; Kairong et al., 1999b)
<i>L. chinensis</i> L.	Shoot tip	(Liu, 1991)
<i>L. halimifolium</i> Mill.	Anther	(Zenkteler, 1972)
<i>Lycopersicon esculentum</i> Mill.	Cotyledon	(Moghaieb et al., 1999)
<i>L. esculentum</i> Mill.	Hypocotyl	(Newman et al., 1996; Moghaieb et al., 1999)
<i>L. esculentum</i> Mill.	Pollen	(Chlyah et al., 1990)
<i>L. esculentum</i> Mill.	Protoplast	(Chen and Adachi, 1994)
<i>L. esculentum</i> Mill.	Seedling	(Gill et al., 1995)
<i>L. esculentum</i> Mill.	Shoot	(Chande and Katiyar, 2000)
<i>L. esculentum</i> x <i>L. peruvianum</i>	Anther	(Chlyah et al., 1990)
<i>L. esculentum</i> x <i>L. peruvianum</i>	Hypocotyl	(Lanzhuang and Adachi, 1996)
<i>L. esculentum</i> x <i>L. peruvianum</i>	Protoplast	(Chen and Adachi, 1998)
<i>L. peruvianum</i> Mill.	Protoplast	(Zapata and Sink, 1981)
<i>L. pimpinellifolium</i> Mill.	Pollen	(Chlyah et al., 1990)
<i>Nicotiana alata</i> Link & Otto	Anther	see (Tisserat et al., 1979)
<i>N. attenuata</i> Torr. ex. Wats.	Pollen	(Collins and Sunderland, 1974)
<i>N. glutinosa</i> L.	Anther	see (Tisserat et al., 1979)

Table 1.1 (continued)

Plant	Explant	Authorities
<i>N. knightiana</i> Good.	Pollen	(Collins and Sunderland, 1974)
<i>N. plumbaginifolia</i> Viv.	Leaf	(Reinbothe et al., 1994)
<i>N. plumbaginifolia</i> Viv.	Protoplast	(Reinbothe et al., 1992)
<i>N. raimondii</i> Macb.	Pollen	(Collins and Sunderland, 1974)
<i>N. rustica</i> L.	Anther	see (Tisserat et al., 1979)
<i>N. sylvestris</i> Spegazzni and Comes	Anther and microspore	see (Tisserat et al., 1979)
<i>N. sylvestris</i> Spegazzni and Comes	Protoplast	(Facciotti and Pilet, 1979)
<i>N. tabacum</i> L.	Anther and microspore	see (Tisserat et al., 1979)
<i>N. tabacum</i> L.	Leaf	(Stolarz et al., 1991; Gill and Saxena, 1993)
<i>N. tabacum</i> L.	Petiole	see (Tisserat et al., 1979)
<i>N. tabacum</i> L.	Protoplast	(Lorz et al., 1977)
<i>N. tabacum</i> L.	Stem	see (Tisserat et al., 1979)
<i>Petunia axillaris</i> (Lam.) B.S.P.	Anther	see (Tisserat et al., 1979)
<i>P. axillaris</i> x <i>hybrida</i> Vilm.	Anther	see (Tisserat et al., 1979)
<i>P. hybrida</i> Vilm.	Anther and microspore	see (Tisserat et al., 1979)

Table 1.1 (continued)

Plant	Explant	Authorities
<i>P. hybrida</i> Vilm.	Leaf	(Rao et al., 1973)
<i>P. hybrida</i> Vilm.	Stem	(Rao et al., 1973)
<i>P. inflata</i> R. Fries	Leaf	(Handro et al., 1973; Rao et al., 1973)
<i>P. inflata</i> R. Fries	Stem	(Handro et al., 1973; Rao et al., 1973)
<i>Solanum aviculare</i> Forst.	Cotyledon	(Alizadeh and Mantell, 1991)
<i>S. carolinense</i> L.	Pollen	(Reynolds, 1990)
<i>S. chacoense</i> Bitt.	Anther	(Cappadocia, 1990)
<i>S. dulcamara</i> L.	Anther	see (Tisserat et al., 1979)
<i>S. melongena</i> L.	Cotyledon	(Saito and Nishimura, 1994; Fari et al., 1995; Sharma and Rajam, 1995a; Farooqui et al., 1997)
<i>S. melongena</i> L.	Hypocotyl	(Kamat and Rao, 1978; Matsuoka and Hinata, 1979; Ali et al., 1991; Sharma and Rajam, 1995a; Sharma and Rajam, 1995b)
<i>S. melongena</i> L.	Leaf	(Gleddie et al., 1983; Rao and Singh, 1991; Rao, 1992; Sharma and Rajam, 1995a; Yadav and Rajam, 1997; Yadav and Rajam, 1998)

**Table 1.1** (continued)

<b>Plant</b>	<b>Explant</b>	<b>Authorities</b>
<i>S. melongena</i> L.	Embryo	see (Tisserat et al., 1979)
<i>S. phureja</i> Juz. & Buk.	Anther	(Veilleux, 1990)
<i>S. sarrachoides</i> Sendt.	Leaf	(Banerjee et al., 1994)
<i>S. tuberlosum</i> L.	Anther	(Sopory et al., 1978; Tisserat et al., 1979)
<i>S. tuberlosum</i> L.	Immature embryo	(Pretova and Dedicova, 1992)
<i>S. tuberlosum</i> L.	Leaf	(Seabrook and Douglass, 2001)
<i>S. tuberlosum</i> L.	Root	(Seabrook and Douglass, 2001)
<i>S. tuberlosum</i> L.	Stem internode	(De-Garcia and Martinez, 1995; Seabrook and Douglass, 2001; Seabrook et al., 2001)

### **1.2.6 Somatic embryogenesis of *Capsicum annuum* L.**

In *C. annuum* L., direct (Harini and Lakshmi Sita, 1993; Jeong et al., 1994; Binzel et al., 1996b; Jo et al., 1996) and indirect somatic embryogenesis (Buyukalaca and Mavituna, 1995; Buyukalaca and Mavituna, 1996; Jo et al., 1996; Mavituna and Buyukalaca, 1996; Kintzios et al., 1998; Kintzios et al., 2000; Kintzios et al., 2001), have been shown to occur.

### ***1.2.6.1 Direct somatic embryogenesis of pepper***

Harini and Lakshmi Sita (1993) first reported direct somatic embryogenesis from immature zygotic embryos of *C. annuum* L. var. California Wonder using the Murashige and Skoog (MS) basal medium supplemented with 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), coconut water and sucrose each at 10% (w/v). Both somatic embryo initiation and maturation were achieved using this single medium. Somatic embryos germinated into plantlets after transfer to the medium containing 2% (w/v) sucrose and 1mg/l gibberellic acid (GA<sub>3</sub>).

Jeong et al. (1994) cultured immature zygotic embryos of hot pepper (*C. annuum* L.) on MS basal medium supplemented with 0.5 to 8 mg/l 2,4-D. Up to 87% of them formed somatic embryos on the plumule without producing an intervening callus. Upon transfer onto MS basal medium, 13% of somatic embryos were converted into plantlets.

Binzel et al. (1996b) applied the direct somatic embryogenesis method to regenerate somatic embryos of *C. annuum* L. cvs. New Mexico - 6 and Rajur Hirapur. The entire process of induction and maturation of the embryos was completed on MS medium containing 2 mg/l 2,4-D, 10% coconut water and 8% (w/v) sucrose. This medium is not too different from that used in the work of Harini and Lakshmi Sita (1993). Binzel et al. (1996b) used a somatic embryo germination medium which is different from that of Harini and Lakshmi Sita (1993) and consisted of MS medium supplemented with 2% (w/v) sucrose, 10 µM silver nitrate (AgNO<sub>3</sub>) and 1 mg/l GA<sub>3</sub> or thidiazuron (TDZ) alone or in combination.

Jo et al. (1996) obtained somatic embryos of red pepper (*C. annuum* L. cv. Nokkwang) starting from 1-2 mm immature zygotic embryo explants. The size of the explants and the concentrations of 2,4-D and sucrose were found to be critical. Somatic embryos were induced directly from the explants and regenerated into plantlets successfully after 3 weeks in the dark on MS medium supplemented with 2 mg/l 2,4-D. For the maturation of the somatic embryos, 0.53 mg/l abscisic acid (ABA) or 20 µM AgNO<sub>3</sub> yielded the best result (25% of explants forming shoot).



### 1.2.6.2 Indirect somatic embryogenesis of pepper

Buyukalaca and Mavituna (1996) first showed regeneration of pepper (*C. annuum* L. var. Ace) through somatic embryogenesis in liquid media. For embryogenic callus formation, mature zygotic embryos were cultured on MS medium with 2 mg/l 2,4-D and 3% (w/v) sucrose. Embryogenic callus was transferred to liquid MS medium with 1 mg/l 2,4-D and 3% (w/v) sucrose to increase the mass of the embryogenic cells. After pretreatment with potassium citrate, cells were placed into embryo initiation medium containing 6 g/l L-proline and a decreased (10 mM) ammonium concentration. Embryos were matured and converted into plants on half-strength MS medium containing 0.5 mg/l ABA.

Jo et al. (1996) obtained somatic embryos of red pepper (*C. annuum* L. cv. Nokkwang starting from 3-4 mm immature zygotic embryo explants. Somatic embryogenic callus was initiated on MS medium containing 5-10 mg/l 2,4-D for 5-7 weeks under continuous illumination. For somatic embryo induction and maturation, the embryogenic callus was transferred to MS medium supplemented with 0.13 mg/l ABA. However, this indirect method was obviously less prominent than direct somatic embryogenesis occurring in the same explants.

Kintzios et al. (1998) and Kintzios et al. (2000) showed the induction, proliferation and development of somatic embryos from young, fully expanded leaves of chilli pepper (*C. annuum* L. cv. Colombo). The leaf explants were cultured either directly on solid MS medium supplemented with 2 mg/l 2,4-D and 2.9 mg/l 6-benzyladenine (BA) or incubated for 24 hours in liquid MS medium containing 2.9 mg/l BA and then transferred to the solid MS medium. Somatic embryos developed and matured on MS medium containing 2% (w/v) sucrose and 1 mg/l GA<sub>3</sub>. Globular embryo proliferation depended on the leaf position on the donor plant: fewer embryos were derived from the third leaf (counting from the base of the shoot) than from the first two leaves. The initial pretreatment of pepper explants with increased BA concentrations significantly reduced the overall proliferation of somatic embryos without affecting the percentage of globular embryos formed which further developed into the torpedo-shape stage and eventually germinated. Depending on the leaf position, somatic embryo induction was significantly affected by the initial culture incubation under illumination or in darkness. Heart- and torpedo-shaped embryos could be observed only on callus pieces initially incubated for 3 weeks in darkness.

Kintzios et al. (2001) also investigated the effect of different vitamins and inorganic micronutrients on callus growth and the induction and proliferation of somatic embryos from young, fully expanded leaves of chilli pepper (*C. annuum* L. cv. Colombo). The results indicated that somatic embryogenesis from pepper leaves is favoured by the addition of nicotinic acid to the culture medium and an increase in copper concentration (an average induction of 70.2 globular embryos/mm<sup>2</sup> of explant surface which is 9.2% higher than control), without affecting embryo maturation and germination.

### 1.2.7 Microscopic studies on somatic embryogenesis

There have been quite a number of experiments attempting to determine the origin and developmental sequence of somatic embryogenesis in higher plants.

Although *Daucus carota* L. is unquestionably a model system for the study of somatic embryogenesis, knowledge about the origin of carrot somatic embryos is still limited (Toonen et al., 1994). Halperin (1966) proposed that indirect somatic embryogenesis of carrot originated from proembryogenic mass (PEM), a clump of small and active superficial cells. They were called embryogenic clumps (EC) by McWilliam et al. (1974) and had large starch grains, a large prominent nucleus, dense cytoplasm and numerous vacuoles. Street and Withers (1974) also found that this group of cells had dense RNA and protein and showed a high level of dehydrogenase activity. Both PEM and EC subsequently developed into globular embryos (Halperin, 1966; McWilliam et al., 1974). Histological studies on somatic embryogenesis in other plants also supported the view that somatic embryos generally seem to originate from small cytoplasm-rich cells (Toonen et al., 1994).

Kohlenbach (1978) suggested two basic events occurring early on during somatic embryogenesis:

- (a) the induction of embryogenic competent cells and
- (b) the development of these embryogenic competent cells to somatic embryos.

In greater details, four different developmental phases have also been described (Komamine et al., 1992):

- (a) State 0. Competent single cell form embryogenic cell clusters,

- (b) State 1. Embryogenic cell clusters proliferate slowly and apparently without further differentiation,
- (c) State 2. Rapid cell division occurs in certain parts of the embryogenic cell cluster, leading to the formation of globular embryos, and
- (d) State 3. Globular embryos develop into plantlets with heart-shaped and torpedo-shaped embryos as the major intermediate stages.

In practice, two distinctly different types of media are generally designed in somatic embryogenesis research: the first medium is designed to induce embryogenic competent cells or activate competent single cells to form embryogenic cell clusters and the second medium is to encourage these embryogenic competent cells or embryogenic cell clusters to develop into somatic embryos. Nonetheless, in some plants both embryoid induction and maturation only require the first medium (Dodds and Roberts, 1987).

de Vries et al. (1988) indicated two pathways for the formation of *Daucus carota* L. embryogenic cells during the initial stage:

- (a) Cells arose from a cell type, susceptible to auxin, which is already present in the explant. This cell type could then develop into a self-perpetuating subpopulation of the embryogenic suspension culture and remain as long as the culture is embryogenic.
- (b) Cells formed *de novo* from previously non-embryogenic single cells or cell clusters present in the suspension culture. The population of embryogenic cells is continuously supplemented by other, non-embryogenic, cell types. It is not known whether one or both pathways could lead to the formation of carrot somatic embryos (Toonen et al., 1994).

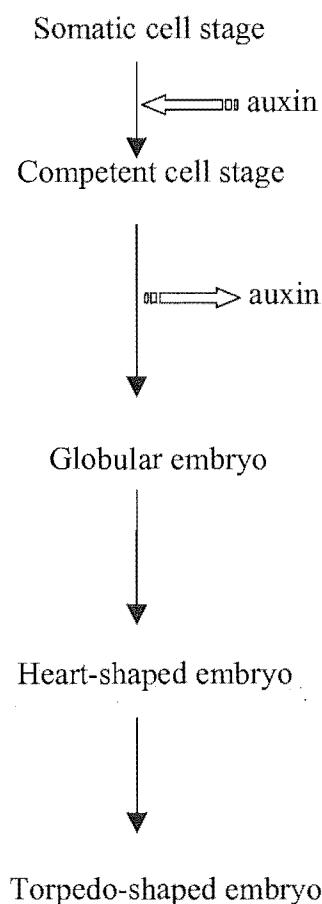
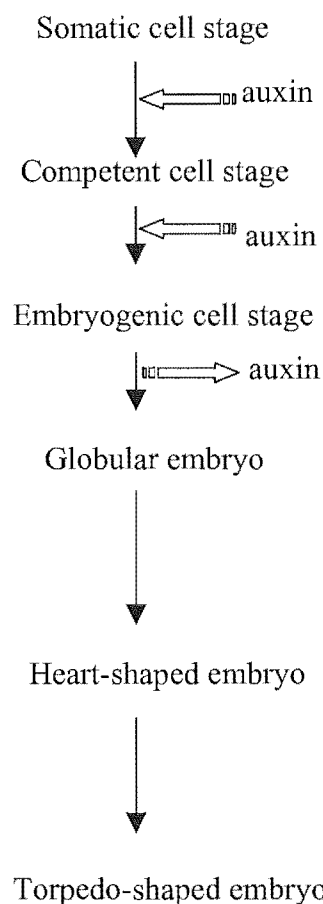
However, the ability to develop somatic embryos may not be limited to one particular cell type. With the video cell tracking system, it was observed that carrot somatic embryos could develop from five cell types: small spherical vacuolated cells; small spherical cytoplasm-rich cells; oval vacuolated cells; elongated vacuolated cells; and irregular shaped cells (Toonen et al., 1994). In particular, both cytoplasm-rich and vacuolated spherical cells appeared and could develop somatic embryos in three distinctive ways. Firstly, asymmetrical cell clusters developed after the first division of oval or elongated single cells. One of the daughter cells developed into a somatic embryo while the other daughter cell expanded and some even divided. This morphologically resembles the

development of the unicellular zygote. Secondly, symmetrical cell clusters developed from spherical vacuolated or spherical cytoplasm-rich cells. Lastly, aberrantly formed cell clusters developed mainly from the irregular-shaped cell types. Basing on these results, Toonen and De Vries (1996) summarised the major steps in somatic embryogenesis as shown in the following schemes (Figure 1.1).

Like carrot, somatic embryogenesis of *Atropa belladonna* L. began with a proembryogenic mass. The embryoids arose in suspension cultures from single cells after unequal divisions. The first division of the original cell was parallel to the long axis and in line with what will be the embryonic axis. This was followed by both anticlinal and periclinal divisions resulting in a small proembryonic mass. Continuous divisions in all planes resulted in a globular embryoid which upon further growth went through heart-shaped and torpedo-shaped stages (Konar et al., 1972).

In leaf and stem culture of *Petunia inflata* R. Fries, all the cytoplasm- and starch-rich cells were recognised as embryogenically competent cells. Embryo development appeared to proceed by periclinal division in an individual cell resulting in the formation of a basal cell and an apical cell. The basal cell underwent another periclinal division while division in the apical cell was anticlinal which was similar to the initial divisions in an egg cell (Handro et al., 1973). Similarly, Matsuoka and Hinata (1979) revealed that embryoids developed as yellowish green spots on the surface of the callus from the hypocotyl of eggplant (*Solanum melongena* L.) after 6 weeks of culture. The callus mass which developed embryoids was soft and watery, and composed of small cell clumps, of which cells were small and rich in cytoplasm.

For direct somatic embryogenesis of *Solanum aviculare* Forst. (Alizadeh and Mantell, 1991), somatic embryos arose in the parenchyma cells associated with the vascular traces closest to the cut basal ends of cotyledonary explants in the dark within the first 3-4 days of culture. Meristematic cells proximal to the first or second vascular traces had numerous plastids with starch and more vacuoles compared with the more distal cells.

Direct somatic embryogenesisIndirect somatic embryogenesis

**Figure 1.1** Pathways of direct and indirect somatic embryogenesis. Embryogenic cells are the cells that have completed the transition from a somatic cell state to one in which no further externally applied stimuli are necessary to produce a somatic embryo (De Jong et al., 1993). The cells that are in this transitional state and have started to become embryogenic cells, but still require externally applied stimuli, are defined as competent cells (Toonen et al., 1994). The somatic cell stage before induction and the competent cell stage requires auxin. The external plant growth regulators are unnecessary for embryogenic cell stage. For direct somatic embryogenesis, somatic cells develop directly into somatic embryos, probably without an intervening, embryogenic cell state (Toonen and De Vries, 1996).

← means with, → without and — shows the direction of development.

Using scanning electron microscope (SEM), Yadav and Rajam (1998) described the following four critical stages of somatic embryogenesis from eggplant leaf discs.

- (a) stage I (the callus-induction phase) between 1 and 6 days of culture leaf disc swelled and then developed into friable callus, initially from the edges and subsequently from other regions of the discs;
- (b) stage II (the cellular acquisition of morphogenetic competence) between 6 and 9 days of culture during which the proembryogenic sectors were formed;
- (c) stage III (the expression of embryogenic program) between 9 and 12 days of culture during which the proembryogenic clusters developed into the first visible globular embryos as green spots; and
- (d) stage IV (the development and maturation of somatic embryos) between 12 and 21 days of culture, during which the globular embryos developed into the heart and then the torpedo stage.

In *C. annuum* L., somatic embryos could apparently form directly on the embryonic axis, cotyledons and shoot apex of the immature zygotic embryo explants (Harini and Lakshmi Sita, 1993; Binzel et al., 1996b). Light microscopy (Jeong et al., 1994) and SEM (Jo et al., 1996) confirmed that direct somatic embryogenesis in *C. annuum* L. exhibited developmental stages similar to those of zygotic embryo development. However, microscopic observation of the early cellular events associated with somatic embryogenesis in *C. annuum* L. is lacking.

### 1.2.8 Regulation of somatic embryogenesis

Somatic cells can be stimulated to become competent for embryogenesis by various means that may include plant growth regulators, pH shock, heat shock and treatment with miscellaneous chemicals (Kohlenbach, 1978; Tisserat et al., 1979; Sharp, 1980; Ammirato, 1983; Bhojwani and Razdan, 1983; Toonen and De Vries, 1996).

The factors which have been reported to be important for induction of somatic embryogenesis were discussed by Tisserat et al. (1979) and the main conclusions are summarised as follow:

- (a) The concentration of some components in culture medium, i.e. nitrogen base, coconut water etc.,
- (b) Physical or environmental condition including light and dark period, light intensity, light quality, temperature and oxygen supply, and
- (c) Source of explants such as nucellus, stem and immature embryo.

Somatic embryo induction often requires a high concentration of an auxin, commonly 2,4-D, in the Murashige and Skoog (1962) (MS) basal medium (Evans et al., 1981). A high level of nitrogen in the form of ammonium nitrate prescribed in the MS medium appears to be especially important (Ammirato, 1983). Cytokinin is usually not required for induction of somatic embryogenesis, although some plant species for example, *Nicotiana tabacum* L. (Gill and Saxena, 1993) and *Zoysia japonica* Steud (Asano et al., 1996), do seem to have a particular requirement for cytokinin. A plant growth regulator-free medium was frequently used for the development of globular embryo into plantlets (Phillips et al., 1995).

#### ***1.2.8.1 Factors important for somatic embryogenesis in Solanaceae***

As mentioned earlier, MS medium has been the most popular medium for somatic embryo induction in Solanaceae for example, Handro et al. (1973); Rao et al. (1973); Kamat and Rao (1978); Facciotti and Pilet (1979); Matsuoka and Hinata (1979); Gleddie et al. (1983); Ali et al. (1991); Alizadeh and Mantell (1991); Liu (1991); Rao and Singh (1991); Stolarz et al. (1991); Saito and Nishimura (1994); De-Garcia and Martinez (1995); Newman et al. (1996); Tu et al. (1996); Cao et al. (1999b); Chande and Katiyar (2000) and JayaSree et al. (2001).

The requirement of plant growth regulators for somatic embryo induction in Solanaceae varies among explants, species or even cultivars. For example, somatic embryo initiation in excised root cultures of *Atropa belladonna* L. var. *lutea* Doll was achieved using 2 mg/l  $\alpha$ -naphthaleneacetic acid (NAA) (Thomas and Street, 1972), while 8 and 10 mg/l NAA were needed in the case of hypocotyl (Matsuoka and Hinata, 1979) and leaf (Gleddie et al., 1983) explants of *Solanum melongena* L., respectively.

Somatic embryogenesis in some plants has been achieved in response to exogenous cytokinin. For example in leaf disc cultures of *Nicotiana tabacum* L. (Gill and Saxena, 1993), thidiazuron (N-phenyl-N'-1, 2,3,-thiadiazol-5-ylurea; TDZ) was effective in inducing somatic embryo formation. For direct somatic embryogenesis in leaf disc cultures of three cultivars of *N. tabacum* L. (Stolarz et al., 1991), some auxin-cytokinin combination such as 1 mg/l BA and 0.1 mg/l NAA was effective. A recent report showed that abscisic acid (ABA) was used to induce somatic embryo formation in *Nicotiana plumbaginifolia* Viv. (Senger et al., 2001). Finally, plant growth regulators may not be always required for somatic embryogenesis (Newman et al., 1996).

Medium components other than plant growth regulators have also been found to influent somatic embryogenesis in Solanaceae. Both  $\text{NH}_4^+$  and  $\text{NO}_3^-$  were found to be essential for embryogenesis from leaf explants of seven *Solanum melongena* L. cultivars on MS medium at optimal ratio of 2:1 ( $\text{NO}_3^-$ :  $\text{NH}_4^+$ ). The optimal sucrose concentration of the medium was 0.06 M and both elevated and reduced sucrose levels inhibited embryogenesis (Gleddie et al., 1983).

Kairong et al. (1999b) discovered an effect of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in somatic embryogenesis of *Lycium barbarum* L.. During the formation of embryogenic cells, there was an increase of intracellular  $\text{H}_2\text{O}_2$ . The highest frequency of somatic embryogenesis was also found in the treatment with 200  $\mu\text{M}$  exogenous  $\text{H}_2\text{O}_2$  for 15 days. However, a higher concentration of  $\text{H}_2\text{O}_2$  (300  $\mu\text{M}$ ) inhibited the formation of somatic embryos.

Sharma and Rajam (1995a and b) discovered genotype, explant and position effects on somatic embryogenesis in four commercially grown Indian cultivars of eggplant: Pusa Purple Long, Long White Cluster, Pusa Kranti, and Pusa Purple Cluster. Moreover, the regulatory role of polyamines in somatic embryogenesis of eggplant was also revealed (Sharma and Rajam, 1995b; Yadav and Rajam, 1997; Yadav and Rajam, 1998).

Recently, the physiological state and size of *Lycopersicon esculantum* Mill. explants at time of culture were found to be critical factors. If the size of explants increased beyond a limit, negative response had been obtained. The optimal size of leaf explant was 0.5  $\text{cm}^2$  and shoot explant was 1 cm long (Chande and Katiyar, 2000).

After the initiation stage, the transfer to auxin-free medium is often sufficient to obtain plantlets from the somatic embryos (Thomas and Street, 1970; Thomas and Street,



1972; Rao et al., 1973; Gosch et al., 1975; Matsuoka and Hinata, 1979). However, plant growth regulators other than auxin may be necessary for somatic embryo maturation and germination. For example, when somatic embryos of *Nicotiana sylvestris* Spegazzni and Comes were transferred to MS medium supplemented with 0.05 mg/l kinetin, they developed further into plantlets (Facciotti and Pilet, 1979).

#### ***1.2.8.2 Factors important for somatic embryogenesis in Capsicum annuum L.***

In pepper, direct somatic embryogenesis from immature zygotic embryo explants seems to require MS medium supplemented with 2 mg/l 2,4-D, 10% coconut water, 8-10% (w/v) sucrose and a photoperiod of 16 hours (Harini and Lakshmi Sita, 1993; Binzel et al., 1996b). The size of immature zygotic embryo explants was also found to be important. The optimal size was approximately 5-8 mm long. While Harini and Lakshmi Sita (1993) indicated that the sucrose concentration is an important factor at the embryo induction stage, Binzel et al. (1996b) concluded that both 2,4-D and cytokinin (presumably supplied via coconut water) were necessary for pepper somatic embryogenesis. However, Jeong et al. (1994) and Jo et al. (1996) induced somatic embryogenesis in pepper immature zygotic embryo explants using a medium free of cytokinin and only 3% (w/v) sucrose. In the latter two studies, 2,4-D was the only plant growth regulator required for somatic embryo initiation and the optimum size of the immature zygotic embryo explants ranged approximately from 0.5 to 4 mm long. Moreover, Jo et al. (1996) found that when the concentration of 2,4-D was raised to 5-10 mg/l, indirect somatic embryogenesis via callus formation was induced under a continuous illumination for 5-7 weeks. Since different studies used different *Capsicum* cultivars, it is possible that genotypic differences may account for the different results obtained.

For somatic embryo germination, Harini and Lakshmi Sita (1993) used MS basal medium supplemented with 1 mg/l gibberellic acid (GA<sub>3</sub>) and 2% (w/v) sucrose. Binzel et al. (1996b) also used MS basal medium but with different supplements: 10 µM AgNO<sub>3</sub> and 0.01 mg/l thidiazuron (TDZ). Jeong et al. (1994) used MS basal medium without any other supplements while Jo et al. (1996) recommended the use of 0.53 mg/l abscisic acid (ABA) or 20 µM AgNO<sub>3</sub>. Although genotypic differences may exist for somatic embryo

germination requirements, there does not seem to be any critical assessment of these requirements.

For indirect somatic embryogenesis of pepper from mature zygotic embryo explants, so far only one group has reported success with *C. annuum* L. var. Ace (Buyukalaca and Mavituna, 1995; Buyukalaca and Mavituna, 1996; Mavituna and Buyukalaca, 1996). Many complicated media were employed to produce plantlets from embryogenic callus. It appears that 2,4-D alone was still required to induce somatic embryos and without pre-treatment with potassium citrate, maturation of somatic embryos could fail. Recently, Kintzios et al. (1998) and Kintzios et al. (2000) obtained yellowish friable embryogenic callus from leaf explants of *C. annuum* L. cv. Colombo on MS basal medium containing 2 mg/l 2,4-D, 2.9 mg/l BA and 8% (w/v) sucrose. The number of globular embryos formed is dependent on the developmental stage of the leaf explants. Moreover, somatic embryos could fully develop when callus pieces were initially incubated in darkness for 3 weeks before transfer to the light condition.

Vitamins and inorganic micronutrients were found to influence callus growth and the induction and proliferation of somatic embryos from leaf explants of *C. annuum* L. cv. Colombo Kintzios et al. (2001). Addition of 0.1 mg/l nicotinic acid and a 10-fold increase of copper concentration in the culture medium resulted in more globular embryo production.

### ***1.2.8.3 Arabinogalactan proteins (AGPs)***

AGPs are proteoglycans widely distributed in the plant kingdom. They are present in cell walls and plasma membranes. Plant cell cultures are known to release them into the medium (van Holst and Clarke, 1985; Komalavilas et al., 1991).

Stacey et al. (1990) studied patterns of expression of the JIM 4 arabinogalactan-protein epitope in cell cultures of carrot. These specific arabinogalactan proteins appear to be essential in carrot somatic embryogenesis. Furthermore, the AGPs from carrot seed extracts have similar gel electrophoretic profiles to those from the embryogenic culture media (Kreuger and van Host, 1993). Kreuger and van Holst (1995) pointed out that the fractionated arabinogalactan proteins from carrot or tomato seeds added to carrot cell lines had a dramatic effect on the carrot cell cultures. Some fractions increased the percentage of

embryogenic cells from about 40% to 80% within one week. Egertsdotter and Von Arnold (1995) found that addition of isolated AGPs from media used for culture of embryogenic cell lines to Norway spruce cell suspensions could either induce somatic embryogenesis or improve the normal development of somatic embryos in non-embryogenic cell lines.

$\beta$ -D-glucosyl Yariv reagent, a chromophoric molecule that particularly binds AGPs has been commonly used to localise AGPs in plant tissue (Serpe and Nothnagel, 1994). Saare et al. (2000) found that the amount of AGPs in embryogenic cultures of *Euphorbia pulcherrima* detected with the  $\beta$ -glucosyl Yariv reagent increased with time. The embryogenic and non-embryogenic callus comprised different sets of AGPs precipitated with the Yariv reagent. When 250  $\mu$ M  $\beta$ -D-glucosyl Yariv reagent ( $\beta$ GlcY) was added to the culture medium used to induce somatic embryogenesis from the root tissues of *Cichorium* hybrid '474', the process was completely inhibited (Chapman et al., 2000). After transfer of the  $\beta$ GlcY-treated roots to medium free of  $\beta$ GlcY, somatic embryo initiation was resumed. Presently, the available data support a role for AGPs in somatic embryogenesis and embryo development.

### 1.3 *In vitro* flowering

*In vitro* flowering has been reviewed previously (Scorza, 1982; Dickens and Van Staden, 1988b). Since the last review, many more reports on flowering *in vitro* have appeared (Table 1.2).

**Table 1.2** List of plant species and different explant types that were reported to form flowers *in vitro*. The plants are classified into family, genus and species according to Judd et al. (1999) and Quattrocchi (2000).

Plant family and species	Type of explants	Authorities
<b>Amaranthaceae</b>		
<i>Amaranthus caudatus</i>	Shoot tip	(Tisserat and Galletta, 1988)

Table 1.2 (continued)

Plant family and species	Type of explants	Authorities
<i>Amaranthus hypochondriacus</i>	Shoot tip	(Tisserat and Galletta, 1988)
<i>Amaranthus gangeticus</i>	Shoot tip	(Tisserat and Galletta, 1988)*
<i>Amaranthus retroflexus</i>	Shoot tip	(Tisserat and Galletta, 1988)*
<i>Amaranthus viridis</i>	Shoot tip	(Tisserat and Galletta, 1988)
<b>Apiaceae (Umbelliferae)</b>		
<i>Ammi majus</i> L.	Cotyledon	(Purohit et al., 1995)
<b>Apocynaceae</b>		
<i>Rauvolfia tetraphylla</i> L.	Shoot tip	(Sarma et al., 1999)
<b>Arecaceae (Palmae)</b>		
<i>Calamus thwaitesii</i> Becc.	Excised embryo	(Ramanayake, 1999)
<b>Araceae</b>		
<i>Pinellia ternata</i> (Thumb.) Breit.	Leaf and stem	(Lee et al., 1988)
<b>Araliaceae</b>		
<i>Panax ginseng</i> C. A. Meyer	Cotyledon	(Tang, 2000)
<i>P. ginseng</i> C. A. Meyer	Cotyledonary node, seedling and zygotic embryo	(Lee et al., 1989) (Lee et al., 1990)*
<i>P. ginseng</i> C. A. Meyer	Petiole	(Lim et al., 1997)
<b>Asclepiadaceae</b>		
<i>Ceropegia jainii</i>	Node	(Patil, 1998)

Table 1.2 (continued)

Plant family and species	Type of explants	Authorities
<b>Asteraceae (Compositae)</b>		
<i>Artemisia annua</i> L.	Juvenile and vegetative part	(Gulati et al., 1996)
<i>Chamomilla recutita</i> L.	Inflorescence	(Kintzios and Michaelakis, 1999)
<i>Cichorium intybus</i> L.	Axillary buds	(Bais et al., 2000; Bais et al., 2001)
<i>C. intybus</i> L.	Root	(Demeulemeester and De Proft, 1999)
<i>Dimorphotheca aurantiaca</i>	Leaf	(Al-Atabee and Power, 1990)
<i>Helianthus annuus</i> L.	Cotyledon, hypocotyl, leaf and shoot tip	(Gerco et al., 1984)
<i>Leptinella nana</i> L	Shoot	(Carson and Leung, 1994a)
<b>Brassicaceae (Cruciferae)</b>		
<i>Brassica napus</i> L.	Cotyledon and hypocotyl	(Koh and Loh, 2000)
<i>Brassica nigra</i> (L.) Koch	Hypocotyl and root	(Mehta et al., 1993)
<i>Brassica oleracea</i> L.	Floret	(Kumar et al., 1995)*
<i>Brassica oleracea</i> L. x <i>Camelina sativa</i> (L.) Crantz	Protoplast	(Hansen, 1998)
<i>Brassica oleracea</i> x <i>Erucastrum abyssinicum</i>	Ovary and ovule	(Sarmah and Sarla, 1998)

Table 1.2 (continued)

Plant family and species	Type of explants	Authorities
<i>Orychophragmus violaceus</i>	Germinated seed	(Luo et al., 2000)
<b>Campanulaceae</b>		
<i>Wahlenbergia stricta</i> L.	Soot tip	(Carson and Leung, 1994b)
<b>Caryophyllaceae</b>		
<i>Dianthus caryophyllus</i> L.	Petal of flower	(Sankhla et al., 1994)
<b>Crassulaceae</b>		
<i>Kalanchoe blossfeldiana</i> Poellniz	Node	(Dickens and Van Staden, 1988a; Dickens and Van Staden, 1990)
<b>Cucurbitaceae</b>		
<i>Cucurmis sativus</i> L.	Cotyledon	(Msikita et al., 1990)*
<i>Momordica charantia</i> L.	Shoot tip	(Wang et al., 2001)
<b>Fabaceae (Leguminosae)</b>		
<i>Arachis hypogaea</i> L.	Base of leaflet	(Chengalrayan et al., 1995)
<i>A. hypogaea</i> L.	Cotyledon	(Kumar and Reddy, 1997)
<i>A. hypogaea</i> L.	Zygotic embryo	(Narasimhulu and Reddy, 1984)*
<i>Glycine max</i> (L.) Merr.	Cotyledonary node	(Jullien and Wyndaele, 1992)
<i>G. max</i> (L.) Merr.	Node	(Dickens and Van Staden, 1987)*
<i>Pisum sativum</i> L.	Cotyledonary node and shoot tip	(Franklin et al., 2000b)*

Table 1.2 (continued)

Plant family and species	Type of explants	Authorities
<i>P. sativum</i> L.	Germinated seed	(Fujioka et al., 1999)*
<i>Vigna mungo</i> (L.) Hepper	Cotyledonary node	(Ignacimuthu et al., 1997)*(Franklin et al., 2000a)
<b>Gentianaceae</b>		
<i>Gentiana manshurica</i> Kitag	Germinated seed	(Zhou et al., 1987)
<i>Gentiana punctata</i> L.	Hairy roots	(Vinterhalter et al., 1999)
<i>Gentiana triflora</i> Pall.	Node	(Zhang and Leung, 2000)
<b>Gesneriaceae</b>		
<i>Streptocarpus nobilis</i> C. B. Clarke	Leaf	(Simmonds, 1987)
<b>Lythraceae</b>		
<i>Cuphea leptopoda</i>	Leaf	(Millam et al., 1997)
<i>C. lutea</i>	Leaf	(Millam et al., 1997)
<i>C. paucipetala</i>	Leaf	(Millam et al., 1997)
<b>Meliaceae</b>		
<i>Melia azedarach</i> L.	Hypocotyl	(Handro and Floh, 2001)
<i>M. azedarach</i> L.	Node	(Thakur et al., 1998)
<b>Orchidaceae</b>		
<i>Cymbidium niveo-marginatum</i> Mak	Shoot	(Kostenyuk et al., 1999)*
<i>Dendrobium candidum</i>	Protocorm or shoot	(Wang et al., 1997)
<i>Doritis pulcherrima</i> x <i>Kingiella</i> <i>philippinensis</i>	Vegetative bud	(Duan and Yazawa, 1994)

Table 1.2 (continued)

Plant family and species	Type of explants	Authorities
<i>Phalaenopsis</i> Pink Leopard ‘Petra’	Floral stalk node	(Duan and Yazawa, 1995)
<b>Poaceae (Gramineae)</b>		
<i>Bambusa arundinacea</i>	Germinated seed	(Nadgauda et al., 1990; Nadgauda et al., 1997)
<i>Bambusa edulis</i>	Node	(Lin and Chang, 1998)
<i>Bambusa vulgaris</i> Schrader ex Wendl.	Node	(Rout and Das, 1994; Rout and Das, 1995)
<i>Dendrocalamus giganteus</i>	Node	(Rout and Das, 1994)
<i>Dendrocalamus hamiltonii</i> Munro	Epicotyl and node	(Chambers et al., 1991)
<i>Dendrocalamus strictus</i> (Roxb.) Nees	Node	(Rout and Das, 1994)
<i>Echinochloa colona</i> (L.) Link	Leaf base and mesocotyl	(Das et al., 1996)
<i>Paspalum notatum</i> Fluegge	Leaf containing the inflorescence	(Luaces and Bovo, 1997)
<i>Pennisetum glaucum</i> (L.) R. Br.	Shoot tip	(Devi et al., 2000)
<b>Podostemaceae</b>		
<i>Polypleurum stylosum</i> (Wight) Hall	Germinated seed	(Sehgal et al., 1993)
<b>Rosaceae</b>		
<i>Fragaria ananassa</i> Duch.	Shoot tip	(Asao et al., 1997)*
<i>Pyrus communis</i> L.	Shoot	(Harada and Murai, 1998)
<i>P. serotina</i>	Shoot tip	(Tsujikawa et al., 1990)



Table 1.2 (continued)

Plant family and species	Type of explants	Authorities
<i>P. serotina</i>	Shoot tip and axillary bud	(Higashiuchi et al., 1990)
<b>Rutaceae</b>		
<i>Citrus limon</i> (L.) Burm. f.	Lateral bud	(Tisserat and Galletta, 1990)
<i>Citrus unshiu</i> Marc.	Lateral bud	(Garcia-Luis et al., 1989; Garcia-Luis and Kanduser, 1995)
<i>Fortunella hindsii</i> (Champ.) Swingle	Internode	(Jumin and Nito, 1996a)
<i>Murraya paniculata</i> (L.) Jack	Internode	(Jumin and Nito, 1996b)
<i>M. paniculata</i> (L.) Jack	Shoot	(Jumin and Ahmad, 1999)
<b>Solanaceae</b>		
<i>Capsicum annuum</i> L.	Shoot tip	(Rogozinska and Tobolewska, 1992)
<i>C. annuum</i> L.	Leaf protoplast	(Saxena et al., 1981)
<i>Capsicum frutescens</i> L.	Shoot tip	(Tisserat and Galletta, 1995)*
<i>Petunia hybrida</i> Hort.	Thin epidermal cell layer	(Mulin and Tran Thanh Van, 1989b)
<i>P. hybrida</i> Hort. x <i>Nicotiana plumbaginifolia</i> Viv.	Thin epidermal cell layer	(Mulin and Tran Thanh Van, 1989a)
<i>Lycopersicon esculentum</i> Mill.	Leaf	(Liu and Li, 1989)
<i>L. esculentum</i> Mill.	Stem segment	(Gamborg, 1995)*
<i>L. pimpinellifolium</i> Brezh.	Leaf	(Liu and Li, 1989)

Table 1.2 (continued)

Plant family and species	Type of explants	Authorities
<i>Nicotiana tabacum</i> L.	Hairy root thin cell layers	(Pasqua et al., 1987)
<i>N. tabacum</i> L.	Thin cell layers of flower stalk or pedicel	(Croes et al., 1986a; Croes et al., 1986b; Barendse et al., 1987; Bridgen and Veilleux, 1988; Smulders et al., 1988; Van der Krieken et al., 1988; Barendse et al., 1989; Van der Krieken et al., 1990; Peeters et al., 1991; Van der Krieken et al., 1991)
<i>Solanum tuberosum</i> L.	Shoot tip	(Al-Wareh et al., 1989)

\* denotes the reports with results on both *in vitro* flowering and fruiting

### 1.3.1 *In vitro* flowering of Solanaceae

Many studies seek to understand the process of *in vitro* flowering of tobacco. The most favourite source of explant is the thin cell layers from a flower stalk or pedicel. Cousson and Tran Thanh Van (1983) cultured thin cell layers excised from the basal segment of flower stalks at the terminal green fruit stage of the inflorescence of *Nicotiana tabacum* L. cv. Samsun on MS basal medium containing 0.2 mg/l indole-3-butyric acid (IBA), 0.215 mg/l kinetin and 3% (w/v) glucose under constant illumination. It was found that a specific quantitative combination of glucose and sucrose could almost substitute for the light requirement. Furthermore, Van den Ende et al. (1984) and Van der Krieken et al. (1988) found that the number of flower buds formed depended mainly on BA level. In combination with NAA, it was the BA concentration which determined whether or not buds were formed and whether or not these buds were borne on an inflorescence. NAA acted in a complex manner: 0.19 mg/l decreased the number of floral buds initiated and

delayed bud emergence but promoted bud outgrowth. Optimal results were obtained when explants were first incubated at a low auxin concentration for 3-5 days before they were transferred to medium with more auxin.

Croes et al. (1985) discovered that the number of tobacco flower buds was greater on tissues from the apical than from the basal portions of floral branches. The capacity to generate these buds was largely determined by tissue age at the stage of the excision. The morphogenetic competence to regenerate flower buds *in vitro* started to decline after anthesis of the attached flower (Croes et al., 1986a; Barendse et al., 1989). Moreover, protein synthesis was studied in relation to *in vitro* flower bud formation in tissue strips of tobacco. The rate of leucine incorporation increased from the 3<sup>rd</sup> day on up to the 7<sup>th</sup> day. Protein synthesis was slow in control explants which did not produce floral buds (Croes et al., 1986b).

The leaf explants of 11 genotypes of *Lycopersicon esculentum* Mill. and *L. pimpinellifolium* Brezh. formed flower buds when cultured on MS medium containing 0.5% NaCl (Liu and Li, 1989). Flower formation occurred both via direct differentiation from the leaf cultures and from regenerated shoots. No flower formation was observed on medium without NaCl or that with 1.0% or more NaCl.

When thin epidermal cell layer explants of *Petunia hybrida* Hort. were excised from floral branches at a specific stage of their development, *in vitro* flowers were formed (Mulin and Tran Thanh Van, 1989b). Floral buds were initiated in 6 weeks from explants excised from the 2<sup>nd</sup> internode while those excised from the 3<sup>rd</sup> one produced only buds after 12 weeks. *In vitro* petunia flowers resemble those formed on plants grown in the greenhouse.

Five-month old plants of *Capsicum annuum* L. cv. California Wonder derived from mesophyll protoplast culture were found to flower *in vitro* under long-day condition (Saxena et al., 1981). The isolated protoplasts were first cultured on medium supplemented with 1 mg/l each of 2,4-D, NAA and BA to produce callus which was then differentiated into shoots on MS medium containing 2.56 mg/l kinetin, 4 mg/l IAA and 3% (w/v) sucrose. For rooting, the MS basal medium was supplemented with 0.04 mg/l kinetin and 1 mg/l IAA.

*In vitro* flowering was induced in 5-mm seedling shoot tip explants of *C. annuum* L. cv. Stano after 6-week incubation on MS medium without any plant growth regulators (Rogozinska and Tobolewska, 1992). This was not observed with other pepper cultivars in the same study. Using a 6-litre polycarbonate container connected to an automated plant culture system (APCS) with liquid medium, flowering *in vitro* from cultured shoot tips of *C. frutescens* cv. 'California Wonder', 'Super Cayenne', and 'Zippy' were observed (Tisserat and Galletta, 1995). These shoots started flowering after 60 to 90 days inside the culture container and flowered continuously thereafter. In contrast, no flowering occurred from shoot tips grown in 25 x 150 mm culture tubes containing agar medium and subcultured every 8 weeks to fresh medium. In all 3 studies on pepper *in vitro* flowering, there was little information about the floral structures.

#### **1.4 *In vitro* fruiting and seed formation**

*In vitro* fruiting of strawberry (*Fragaria ananassa* Duch. cvs. Toyonoka and Summerburry) was observed after hand pollination of flowers produced in shoot apex culture using modified liquid MS basal medium supplemented with 0.5 mg/l BA in a culture-bottle (100 mm X 110 mm X 100 mm) (Asao et al., 1997). *In vitro* pod formation in groundnut (*Arachis hypogaea* L.) occurred when the cotyledon explants were cultured on modified MS basal medium consisting of 0.5 mg/l kinetin in complete darkness. No *in vitro* floral buds were formed if these explants were cultured on medium supplemented with IAA, NAA, GA<sub>3</sub> or ABA alone (Narasimhulu and Reddy, 1984). Tomato (*Lycopersicon esculentum* Mill.) plantlets from clonal propagation of the stem segments were also found fruiting after treatment of the *in vitro* flowers with NAA. However, these mature fruits did not contain seeds (Gamborg, 1995). In contrast, plant growth regulator was not necessary for the *in vitro* fruiting of soybean [*Glycine max* (L.) Merrill. cv. Impala]. The nodal explants of this short-day cultivar could produce 100% fruiting on the modified plant growth regulator-free MS basal medium, modified to contain no NH<sub>4</sub>NO<sub>3</sub> and a fifth strength of all the other inorganic salts under short day condition (Dickens and Van Staden, 1985).

Fujioka et al. (1999) also found that *in vitro* pod setting of pea (*Pisum sativum* L. cv. Kishu-usui) could be achieved using plant growth regulator-free MS basal medium

supplemented with 3% sucrose. The highest percentage of pod setting was seen when larger containers (30 X 200 mm test tube) and cotton stoppers were used. Only one seed per pod was found. Furthermore, the highest number of seeds per pod in cauliflower (*Brassica oleracea* var. botrytis) was  $4.6 \pm 0.5$  when the curd explants were cultured on MS basal medium supplemented with 3 mg/l IAA and 0.5 mg/l kinetin (Kumar et al., 1995).

Viability of *in vitro* seeds was reported by Ignacimuthu et al. (1997) in black gram [*Vigna mungo* (L.) Hepper cv. Vamban] when nodal explants were cultured on MS basal medium consisting of half strength inorganic salts, full strength organic compounds, 0.1-0.5 mg/l IBA and 3% sucrose. No *in vitro* flowers were formed when the explants were cultured on full strength MS medium. There was no information about the germination of the *in vitro* seeds. Recently, Franklin et al. (2000) reported that *in vitro* seeds of green pea (*Pisum sativum* L. cv. PID) could germinate. The highest number of pods formed occurred when the shoot explants were cultured on modified MS basal medium containing half strength  $\text{NH}_4\text{NO}_3$ , 1 mg/l  $\text{GA}_3$ , 0.5 mg/l IBA and 3% sucrose.

In *Capsicum frutescens* L. cv. California Wonder, *in vitro* fruiting occurred when the shoot tip explants were cultured in a 6-litre polycarbonate container connected to an automated plant culture system providing liquid MS basal medium consisting of 0.5 mg/l thiamine, 100 mg/l i-inositol and 3% sucrose (Tisserat and Galletta, 1995). About 5% to 10% of the flowers set fruits. Maximum fruit size obtained was about 25% to 75% of the size of fruit produced on those plants grown in the glasshouse. *In vitro* fruiting was possible in this study probably because improved gas exchange using the APCS air exchange system. If the air supply was not connected to the culture container, *in vitro* fruiting was not observed. It was not reported whether there were any viable or germinable seeds inside the *in vitro* fruits.

### 1.5 *In vitro* propagation

Since most flowering plants are highly heterozygous, their seed offsprings are not true-to-type. On the contrary, asexual reproduction or vegetative propagation should give rise to plants that are genetically identical to the parent plant and thus permit the continuation of

the unique characters of the cultivars. Multiplication of plants starting from a single individual or explant of a cultivar by asexual reproduction or vegetative propagation is called clonal propagation (Bhojwani and Razdan, 1983; Phillips and Gamborg, 1995).

The process of *in vitro* propagation proceeds through a series of steps, each with a specific set of requirements:

- (a) selection, preparation and pretreatment of the suitable explants;
- (b) initiation of aseptic cultures by sterilisation and transfer to nutrient medium;
- (c) proliferation of shoots on multiplication medium;
- (d) transfer of shoots to a rooting (or storage) medium; and
- (e) hardening for subsequent field planting (Murashige, 1974; Bhojwani and Razdan, 1983; Hussey, 1983; Rice et al., 1992; Dhawan, 1993).

The use of *in vitro* techniques for *in vitro* propagation is the most advanced application of plant tissue culture. This can be achieved by (1) shoot or axillary bud culture, (2) production of adventitious buds, and (3) somatic embryogenesis. In the latter two methods, organised structures arise directly on the explant or indirectly from callus. Shoot or axillary bud produces the least number of plantlets as the number of new shoots produced is controlled by the number of shoots or axillary buds cultured, but remains the most widely used method in commercial practices and produces the most true-to-type plantlets. For biotechnology, axillary shoot proliferation is used as a supporting technology at both the beginning and the end of the process. At the beginning the *in vitro* stems and leaves serve as a source of explants and, at the end, axillary cultures are used to propagate clonally the special plants that are the result of the biotechnological manipulations. Adventitious budding has a greater potential for producing plantlets, as bud primordia may be formed on any part of the explants. Adventitious shoots or roots can be induced to form on tissues which normally do not produce these organs. This process is much more common than somatic embryogenesis and has far more potential for mass clonal propagation of plants than multiplication from shoot or axillary buds. Unfortunately, somatic embryogenesis, which has the potential of producing the largest number of plantlets, has not yet to be applied commercially to any great extent (Binding and Krumbiegel-Schroeren, 1984; Thorpe and Patel, 1984; Brown and Thorpe, 1995; Preece, 1997).

An advantage of axillary shoot proliferation over production of adventitious shoots is the less likelihood of encountering somaclonal variation. Axillary shoots form from preformed meristems at nodes. The chances of the organised shoot meristem undergoing a mutation is relatively low. Therefore axillary shoot proliferation is used to propagate plants clonally. Somaclonal variation is more likely to occur in regeneration system via callus (Preece, 1997).

### **1.5.1 Practical importance of clonal propagation**

The purpose of clonal propagation is to make multiple copies of the plants with desirable qualities (Murashige, 1989). Clonal propagation may be applied in various ways as described below:

- (a) enabling rapid genetic improvement of superior genotypes. Clonal propagation enables retaining most of the genetic potential of elite or selected plantations, including the non-additive components of genetic variance in new generations.
- (b) increasing multiplication rate. Theoretically, the potential exists for a thousand- or even a million-fold increase in the rate of clonal propagation over conventional propagation methods.
- (c) propagation of heterozygous, sexually incompatible, and sterile genotypes. These characteristics occur in many ornamental, vegetable, and fruit crops;
- (d) maintenance of elite plants since rooting stem and leaf cuttings is slow and inefficient;
- (e) maintenance of inbred parent lines for F1 hybrid seed production;
- (f) bulking up haploid and doubled-haploid plants for breeding;
- (g) replacing the expensive nursery practices such as grafting or budding on a rootstock;
- (h) cloning of spontaneous or induced mutants;
- (i) permitting multiplication of herbaceous plants all year round without possible limitations associated with seasons;
- (j) facilitating the creation of germplasm banks for preservation of valuable plant materials and their storage under pathogen-free conditions in a relatively small space; and

(k) *in vitro* cloning disease-free plants for phytosanitary transport from country to country (Conger, 1981a; Conger, 1981b; Bhojwani and Razdan, 1983; Redenbaugh, 1991; Pierik, 1993; Muralidharan and Mascarenhas, 1995; Jain, 1999).

### 1.5.2 Clonal propagation of *Capsicum* spp.

A summary of the major relevant studies on clonal propagation of *Capsicum* spp. is given in Table 1.3.

**Table 1.3** List of the *Capsicum* species and type of explants for clonal propagation. The plants are classified into species according to IBPGR (1983).

<i>Capsicum</i> species	Type of explants	Authorities
<i>Capsicum</i> sp. (sterile mutant)	Shoot tip	(Sultanbawa and Phatak, 1991)
<i>C. annuum</i> L. cvs. Beldi, D'hirat and Semmane	Stem node	(Chebchoub, 1987)
<i>C. annuum</i> L. cv. Bhivapuri	Shoot meristem	(Madhuri and Rajam, 1993)
<i>C. annuum</i> L. cv. G4	Shoot tip	(Christopher and Rajam, 1994)
<i>C. annuum</i> L. cv. Early California Wonder	Shoot tip	(Ebida and Hu, 1993)
<i>C. annuum</i> L. cv. Mathania	Shoot tip	(Agrawal et al., 1988)
<i>C. annuum</i> L. cv. Soroksari	Shoot tip	(Berljak, 1999)
<i>C. annuum</i> L. (4 cvs.)	Shoot tip	(Phillips and Hubstenberger, 1985)
<i>C. annuum</i> L. (4 cvs.)	Shoot tip	(Morone-Fortunato and Tudisco, 1991)



Table 1.3 (continued)

<i>Capsicum</i> species	Type of explants	Authorities
<i>C. annuum</i> L. (6 cvs.)	Shoot tip	(Rogozinska and Tobolewska, 1992)
<i>C. annuum</i> L. (11 vars.)	Shoot tip	(Fari and Csillery, 1983)
<i>C. annuum</i> x <i>C. chinense</i> (6 genotypes)	Shoot tip	(Fari and Csillery, 1983)
<i>C. baccatum</i> L. vars. pen-1 and lut-1	Shoot tip	(Fari and Csillery, 1983)
<i>C. chacoense</i> A.T. Hunz. var. cha-1	Shoot tip	(Fari and Csillery, 1983)
<i>C. chinense</i> Jacq. (18 genotypes)	Shoot tip	(Fari and Csillery, 1983)
<i>C. eximium</i> A.T. Hunz. var. exi-5	Shoot tip	(Fari and Csillery, 1983)
<i>C. frutescens</i> L. (3 genotypes)	Shoot tip	(Fari and Csillery, 1983)
<i>C. frutescens</i> L. (3 cvs.)	Shoot tip	(Tisserat and Galletta, 1995)
<i>C. praetermissum</i> Heiser & Smith (PI 342947)	Shoot tip	(Christopher and Rajam, 1994)
<i>C. praetermissum</i> Heiser & Smith var. pra-7	Shoot tip	(Fari and Csillery, 1983)
<i>C. pubescens</i> R. & P. (3 genotypes)	Shoot tip	(Fari and Csillery, 1983)

Although Fari (1986) mentioned that a reliable method for the effective meristem culture of pepper had not been found, Madhuri and Rajam (1993) reported that multiple shoots (5-7 per explant) could be produced from the apical shoot meristem of approximately 0.8 mm long of *Capsicum annuum* L. on a filter paper bridge in liquid MS medium supplemented with 2 mg/l BA. For nodal culture, there was only one report in which nodes from adult plants of *C. annuum* L. were cultured on modified MS medium

supplemented with the 0.1 mg/l NAA and 0.5 mg/l BA or 0.1 mg/l NAA, 0.5 mg/l BA and 0.5 mg/l kinetin or only 0.5 mg/l BA (Chebchoub, 1987). Shoots developed and rooted within 5 weeks. The axillary buds from these shoots could be regenerated again into shoots and rooted as well.

So far *in vitro* clonal propagation through shoot-tip culture has not been successfully reported in *C. annuum* L. as none of these genotypes persisted after the second or third transfer (Fari and Andrasfalvy, 1994; Ezura, 1997). Fari and Csillery (1983) used 6 different types of modified MS and Nitsch (1968) media with or without plant growth regulators to culture shoot tips of eight *Capsicum* species *in vitro*. It was found that only the cloning ability (i.e. growing of excised shoot-tips for extended times) was shown by some genotypes, thus suggesting that this trait may have a genetic basis (Fari and Andrasfalvy, 1994).

When shoot tips of *C. annuum* cvs. California Wonder, Yolo Wonder, New Mexico No. 6-4 and NuMex R. Naký were cultured on MS basal medium containing 0.05 mg/l IAA and 0.05 mg/l BA, shoot elongation was promoted. While at a higher concentration (0.05-4 mg/l IAA and 10-50 mg/l BA), adventitious shoot bud formation was promoted. Raising the incubation temperature to 28.5 °C in continuous light resulted in the best shooting response (87.5%). Glucose was also found to be superior to sucrose as the carbon source. Shoot tips generally exhibited a 2- to 10-fold greater capacity for shoot organogenesis compared to hypocotyl and cotyledon explants (Phillips and Hubstenberger, 1985).

MS medium supplemented with 5 mg/l BA was found to be most effective for shoot bud differentiation from shoot tip explants of *C. annuum* L. cv. Mathania (Agrawal et al., 1988; Agrawal et al., 1989). The basal end of the explant callused and 7-12 shoot buds were formed after 2 weeks. Elongation of shoot tip explants was found in the presence of low levels of BA alone or in auxin-cytokinin combinations. However, there was no instance whereby the elongation of the parent shoot was observed in a plant growth regulator-free medium. These shoot buds after being cultured on 5 mg/l BA increased in number but did not elongate. To obtain complete plantlets, shoot buds were transferred to a medium with 0.1 mg/l IBA or 0.1 mg/l NAA. Direct differentiation of shoot buds in response to BA or kinetin was not observed in stem nodal explants. Similar results were obtained using shoot-tip explants of *C. annuum* cvs. Rosso d'Asti, Tondo

liscio, Corno di toro rosso o giallo and Verde piccolo per sottaceto (Morone-Fortunato and Tudisco, 1991), and Early California Wonder (Ebida and Hu, 1993).

Sultanbawa and Phatak (1991) discovered that 60% of 2-3 cm long shoot tips excised from a sterile (nonflowering) mutant pepper plant (*Capsicum* sp.) with large, purplish-green, leathery leaves and purplish-green stems rooted *in vitro* after 8 weeks of culture on half-strength MS medium without any plant growth regulator. Media that contained 1 or 2 mg/l IBA induced callusing but not rooting.

In *C. praetermissum* Heiser & Smith and *C. annuum* L. (Christopher and Rajam, 1994), high level of BA (15 mg/l) or kinetin (20 mg/l) were essential for maximum shoot proliferation from shoot-tip explants after 4 weeks of culture. Combination of 1  $\mu$ M 2, 3, 5-triiodobenzoic acid (TIBA) with lower levels of BA or kinetin significantly increased shoot number as compared to using either cytokinin alone. Best rooting (80-100%) of regenerated shoots was achieved on MS medium containing TIBA in combination with either BA or kinetin. The resultant plantlets were normal diploids while those obtained on medium without TIBA but with BA or kinetin alone showed distinct chromosomal aberrations. However, lower levels of BA could initiate direct shoot regeneration from basal part of shoot-tip explants of *C. annuum* L. cv. Soroksari when the explants were cultured on modified MS medium supplemented with and 2 mg/l BA and 0.5 mg/l IAA or 1 mg/l BA or 2 mg/l zeatin (Berljak, 1999). It seems that different cultivars may have different plant growth regulator requirements.

More recently, Tisserat and Galletta (1995) reported another benefit of clonal propagation using shoot-tip explants. They found that flowering and fruit production were obtained from cultured shoot tips of *C. frutescens* cvs. California Wonder, Super Cayenne, and Zippy grown in a liquid MS medium without plant growth regulator in a 6-litre polycarbonate container connected to an automated plant culture system (APCS). However, no flowering appeared from shoot tips which were in 25 x 150 mm culture tubes subcultured every 8 weeks to fresh MS agar medium.

## 1.6 Aim and objectives

The application of plant tissue culture to obtain a better understanding of several developmental processes in *Capsicum annuum* L. is of central interest in this research. From the literature review, there are several reports on somatic embryogenesis, two reports on *in vitro* flowering, and one report on *in vitro* fruiting in a few cultivars of *C. annuum* L. using different explants. There has been not a single study using one type of explant from one cultivar to investigate the important factors influencing developmental processes from somatic embryogenesis to *in vitro* flowering, fruiting and seed production. Here, the immature zygotic embryo explants of the cultivar Sweet Banana were used for this research problem. Some of the experiments on the cv. Sweet Banana were also repeated using some other cultivars including cvs. California Wonder, Yolo Wonder and Ace. A major consideration in choosing these cultivars was that their seeds were commercially available.

This research began with an evaluation of several published protocols for somatic embryogenesis in *C. annuum* L. to see if they can be applied to cultivars other than those used in the previous reports. This led to a re-examination of some of the important factors influencing somatic embryogenesis in some of the *Capsicum* cultivars used in this study. A histological study was also carried out to lend support to the contention that somatic embryos were indeed obtained using the modified protocols described here. The plantlets regenerated via somatic embryogenesis were used in several ways. Firstly, experiments were carried out to determine if the somatic embryo-derived plantlets could be established in soil, grown in the glasshouse and eventually set fruits with germinable seeds. The data are important to see if the process of somatic embryogenesis has any undesirable effects. Secondly, experiments starting with the somatic embryo-derived plantlets were designed to test the hypothesis that the whole life cycle of a pepper plant can be completed under *in vitro* condition in a small tissue culture container. Thirdly, experiments toward clonal propagation of the somatic embryo-derived plantlets were carried out. The ultimate goal here was to have more copies of the somatic embryo-derived plantlets so that the previously stated hypothesis could be tested in greater depth.

## **Chapter 2**

# **Materials and Methods**

### **2.1 Sources of chemicals and seeds**

All chemicals used to prepare stock solutions of MS basal medium (Murashige and Skoog, 1962) were purchased from BDH Laboratory Supplies, Poole, England, while coconut water and plant growth regulators were obtained from SIGMA<sup>®</sup> Chemical Co., St Louis, USA. Agar was supplied by Germantown company, New Zealand.

Seeds of sweet pepper, *Capsicum annuum* L. cvs. Sweet Banana, California Wonder and Yolo Wonder 'B' were obtained from Arthur Yates & Co Ltd., New Zealand. Those of cv. Ace were obtained from Unwins Seeds Ltd, Histon, Cambridge, England.

Some horticultural characteristics of interests about these cultivars are given in Appendix 3.

### **2.2 Evaluation of *C. annuum* L. somatic embryogenesis protocols**

#### **2.2.1 Attempt to induce somatic embryos using mature seed explants**

To investigate the feasibility of somatic embryogenesis from mature pepper seed, an initial experiment was carried out following the procedure of Buyukalaca and Mavituna (1996) who first described regeneration of pepper (*C. annuum* L. var. Ace) via somatic embryogenesis from mature seed explants using liquid media.

Mature dry seeds of *C. annuum* L. cvs. Sweet Banana, California Wonder and Yolo Wonder 'B' were immersed in distilled water overnight before they were surface sterilised by soaking in 1% (v/v) sodium hypochlorite for 10 minutes and then rinsed three times

with sterile distilled water. After this the seeds were aseptically dissected to obtain the embryo explants which were placed in polycarbonate tissue culture containers of 8.5 cm high x 6.5 cm diameter each with 45 ml medium. The protocol of Buyukalaca and Mavituna (1996) involving the following sequence of media was used without modification: (1) embryogenic callus formation, mature zygotic embryos were cultured on MS basal medium (Murashige and Skoog, 1962; also see Appendix 1 for details) supplemented with 2 mg/l 2,4-D and 3% (w/v) sucrose; (2) subculture of embryogenic callus in liquid MS basal medium supplemented with 1 mg/l 2,4-D and 3% (w/v) sucrose; (3) pretreatment in liquid MS basal medium (KNO<sub>3</sub> free) supplemented with 6 g/l potassium citrate, 2 mg/l 2,4-D and 3% (w/v) sucrose for 3 weeks; (4) embryo initiation in liquid MS basal medium supplemented with 6 g/l L-proline, 10 mM NH<sub>4</sub>NO<sub>3</sub> and 3% (w/v) sucrose for 3 weeks; (5) maturation of embryos in half-strength liquid MS basal medium supplemented with 0.5 mg/l ABA and 3% (w/v) sucrose under dark condition; and (6) conversion into plantlets on half-strength MS basal medium supplemented with 0.5 mg/l ABA and 3% (w/v) sucrose in the light.

Unless specified otherwise, all media in this study were adjusted to pH 5.7, gelled with 0.8% (w/v) agar (Germantown company, New Zealand) and autoclaved at 121°C and 15 *psi* for 20 minutes.

## **2.2.2 Immature zygotic embryo explants**

### **2.2.2.1 Growing *C. annuum* *L.* plants**

For production of immature seeds, plants of the 4 cultivars of pepper (cvs. Sweet Banana, California Wonder, Yolo Wonder 'B' and Ace) were grown separately in the glasshouses at the University of Canterbury until flower formation, fruit setting and seed production (Plate 2.1). A potting mix with a slow releasing fertiliser (see Appendix 2.3) lasting for 8-9 months was used. If required, flowers were labelled on the day of their opening (also see Appendix 2).

**Plate 2.1** Plants of the 4 cultivars of *Capsicum annuum* L. grown separately in the glasshouses at the University of Canterbury.

- (a) cv. Sweet Banana
- (b) cv. California Wonder
- (c) cv. Yolo Wonder 'B'
- (d) cv. Ace





#### ***2.2.2.2 Selection of immature zygotic embryo explants***

In the protocols of Harini and Lakshmi Sita (1993) and Binzel et al. (1996), the fruits were collected from field- or greenhouse-grown plants before they were surface sterilised directly. After this immature zygotic embryos were isolated from the seeds of the sterilised fruits. Most of the zygotic embryos were visually judged to be not of the right size (5-7 mm) and had to be discarded. Those of the right size would then be placed on medium.

In the present study, several (5-6) immature pepper fruits of a cultivar were cut outside on a lab bench within an hour after they were collected from the glasshouse-grown plants. The seeds of a fruit were placed in a plastic Petri dish (9 cm diameter). Only a few seeds were selected randomly from each fruit for embryo isolation. After this the immature zygotic embryos were assessed with the aid of a ruler to see if most of them were of the appropriate size (see 3.2.1) for somatic embryogenesis experiments. If so, the rest of the seeds from the same fruit would be chosen for surface sterilisation. If many of the selected seeds had inappropriate size, the seeds from that fruit would be discarded and not used in the present experiments. Much time, particularly in surface-sterilising seeds that were not appropriate for further experiments, had been saved using the present seed selection method over the previously published protocols.

#### ***2.2.2.3 Surface sterilisation***

Seeds were surface sterilised in a laminar flow hood by soaking in 1% (v/v) sodium hypochlorite for 7 minutes before they were rinsed three times with sterile distilled water. The duration of surface sterilisation was important. If it was too short, the culture would easily get contaminated. If it was too long, the immature zygotic embryo explant would die.

The isolation of the explants from the seeds under aseptic conditions was also difficult. If the seed was pushed too hard, the explant would be damaged. If this happened, it would form fewer somatic embryo structures or die. The best way to excise the immature zygotic embryo explant was first gently remove the seed coat and endosperm. The seed coat was peeled starting from the hilum end towards the opposite site using one pair of forceps while keeping the seed vertical using another pair of forceps. Then, the seed coat

was left at the side of a Petri dish. A slight pressure, first around the radicle end and then the cotyledon end, would free the embryo from the endosperm.

The embryo explants were placed in polycarbonate tissue culture containers (LabServ, Auckland, New Zealand) of 8.5 cm high x 6.5 cm diameter each with 45 ml medium. Unless specified otherwise, all cultures in this study were kept in a growth room at 22 °C under continuous illumination (average 26.5  $\mu\text{E}/\text{sec}/\text{m}^2$ , measured by 21X Micrologger, Campbell Scientific Inc., USA) provided by white fluorescent lamps.

#### ***2.2.2.4 Media of published protocols***

For somatic embryo induction, immature zygotic embryos of 4 *Capsicum* cultivars were cultured on MS basal medium supplemented with 2 mg/l 2,4-D, 10% (w/v) each of coconut water (Sigma, St. Louis, USA) and sucrose. After 6 weeks, all explants with somatic embryo structures were transferred to MS basal medium containing 2% (w/v) sucrose and 1 mg/l GA<sub>3</sub> for germination. Both media for somatic embryo induction and germination were those previously described by Harini and Lakshmi Sita (1993).

In addition, the protocol of Binzel et al. (1996) for somatic embryo germination was also tried. This medium consisted of MS basal medium with 2% (w/v) sucrose, 10  $\mu\text{M}$  AgNO<sub>3</sub> and 1 mg/l GA<sub>3</sub>.

#### ***2.2.2.5 Modified media***

In the present research, immature zygotic embryo was isolated aseptically and was then placed on induction medium (MS basal medium supplemented with 2 mg/l 2,4-D and 10% (w/v) sucrose) which was essentially the same as that of Harini and Lakshmi Sita (1993) except without 10% (w/v) coconut water. Somatic embryo structures were then transferred to germination or conversion medium (MS basal medium containing 1 mg/l GA<sub>3</sub>, 20  $\mu\text{M}$  AgNO<sub>3</sub> and 2% (w/v) sucrose) which was the same as that of Binzel et al. (1996) except the increased concentration of AgNO<sub>3</sub> used here. The plantlet development medium contained MS salt and vitamin mixture supplemented with 1 mg/l NAA without any sugar. These media were used in the experiments described in 2.3 to 2.7.

## **2.3 Critical factors influencing somatic embryogenesis from immature zygotic embryo explants**

### **2.3.1 Time required on the induction medium**

In this research, the effect of incubating immature embryos from 2 cultivars, Sweet Banana and California Wonder, on the induction medium for 2 or 4 weeks before they were transferred to the germination or conversion medium was studied.

### **2.3.2 Requirement for coconut water in the induction medium**

The effect of coconut water, a source of cytokinin, was claimed to be more effective for induction of somatic embryogenesis (Binzel et al., 1996). This was investigated here using immature zygotic embryo explants from 4 cultivars, Sweet Banana, California Wonder Yolo Wonder, and Ace. The explants were left on the induction medium with or without 10% coconut water for about 2 weeks before they were transferred to the germination or conversion medium.

### **2.3.3 Effect of light and dark condition at the induction stage**

Immature zygotic embryo explants from 3 cultivars, Sweet Banana, California Wonder and Yolo Wonder, were placed on the induction medium in the light or in the dark for about 2 weeks. Then, the explants were transferred to the germination or conversion medium.

### **2.3.4 Effect of different sugar types**

To study the effect of different sugars in the induction medium on pepper somatic embryogenesis, immature zygotic embryo explants from cv. Yolo Wonder were placed for 2 weeks on induction medium without any sugar or supplemented singly with 10% (w/v) of the following sugars: glucose, fructose, maltose and sucrose. Then, the explants were transferred to the germination or conversion medium.

### **2.3.5 Replacement of coconut water with BA**

An experiment was carried out to replace coconut water in the induction medium with 2.9 mg/l BA. Immature zygotic embryo explants from cvs. Sweet Banana, California Wonder and Yolo Wonder were cultured on the BA containing medium for about 2 weeks before they were transferred to the germination or conversion medium.

### **2.3.6 Seasonal effect**

The data on the characteristics of selected fruits and seeds from the plants (*C. annuum* L. cv. 'Sweet Banana') grown in the glasshouse at the University of Canterbury and their possible relationships with somatic embryogenesis were collected during Summer, Autumn, Winter and Spring over 3 years.

It is important to note that although the 4 cultivars of pepper were grown in the glasshouses at the University of Canterbury, the control of temperature was less than desirable and varied from season to season (see Appendix 4). Most experiments in this thesis were mostly done during summer and autumn months because it was more difficult to obtain a sufficient number of appropriate embryo explants in spring and winter. Also the percentages of induction and conversion were significantly higher using explants from fruits harvested in summer and autumn (see Appendix 5).

### **2.3.7 Effect of Yariv's reagent**

Yariv's reagent ( $\beta$ GluY), which has specific affinity for arabinogalactan proteins, was made up as described previously (Yariv et al., 1962). Somatic embryo induction medium was supplemented with or without 20  $\mu$ g/ml of Yariv's reagent. The immature zygotic embryo explants of *C. annuum* L. were cultured on these media for about 2-3 weeks before they were transferred to the germination or conversion medium.

## **2.4 Histology of somatic embryogenesis**

Plant microtechnique (specimen preparation for resin sectioning) was used to examine the origin of the somatic embryo structures obtained (also see Appendix 6).

### 2.4.1 Glutaraldehyde fixation

Zygotic embryos of *C. annuum* L. cv. Sweet Banana from day 0, 3, 5, 7, 10, 14 and 16 on induction medium and somatic embryos from day 3, 5, 7 and 10 on germination or conversion medium (referred to as 'sample' here) were taken out from the tissue culture containers. The samples were dissected in 0.075 M phosphate buffer (pH 7.2) or distilled water to stop any air bubbles from getting into the tissue. If the sample was large, it would be cut into pieces approximately 5 mm cubed. Once cut, the sample was immediately placed into vials of glutaraldehyde fixative [3 % (v/v) glutaraldehyde in 0.075 M phosphate buffer (pH 7.2)].

Once in the fixative the samples were pumped down in a vacuum desiccator to remove all the air from the tissue and allow the fixative to penetrate all the tissue. After this the samples were kept under vacuum overnight. The samples were stored in a refrigerator until use.

### 2.4.2 Dehydration

The samples were dehydrated in the vials with a graded ethanol series: 50, 70, 80, 95, and 100% (v/v) for at least 1 hr. Then they were transferred into 100% ethanol again and was kept overnight.

### 2.4.3 Resin infiltration

The absolute ethanol was replaced by infiltration with resin of hydroxyethyl-methacrylate (Kulzer and Co., GmbH, Wehrheim, Germany). The infiltration solution was prepared from a Technovit® 7100 Resin kit, which contained the hydroxyethyl-methacrylate resin base plus catalyst 'hardener I'. During this period, the samples were infiltrated at 4°C for at least 2 weeks (can be stored up to 4 weeks). The samples and resin were gently rotated (to allow even coverage) every 2 days. When infiltrated with resin, the sample should be semi-transparent.

#### 2.4.4 Resin Embedding

The 0.25 ml plastic mould was filled with embedding resin, a mixture of the fresh infiltration solution (same as section 2.4.1.3) and 1/15 (v/v) 'hardener II'. Then the samples were transferred into the moulds of resin and adjusted into the required sectioning position. The samples were left until the resin polymerised (at least 1 hour).

After polymerisation, support stubs for the moulds were made from gelatine capsules (long half part) and hard support resin, Technovit® 3040 Resin Cold-curing for direct surface testing and impressions (Kulzer and Co., GmbH, Wehrheim, Germany). This resin was mixed, and poured into the gelatine capsule up to a level of 2 mm above the top. After this the plastic mould with a sample was immediately placed upside down on the top of a gelatine capsule (resin surface to resin surface). Fusion of the two resins forms a solid joint between stub and sample block. After 5-10 minutes, the plastic moulds were removed from the sample blocks which were then ready for sectioning.

#### 2.4.5 Sectioning

A Ralph type glass knife made on an LKB 2078 HistoKnifemaker (LKB-Produkter AB, Sweden) was put into the knife-holder and firmly locked on Reichert-Jung 2040 Autocut Rotary microtome (Cambridge Instruments GmbH, Germany). Slides were cleaned in 70% ethanol and dried. Distilled water was placed in drops onto the slide. The sample block was trimmed if there was excess resin surrounding the specimen. The sample block was placed into the specimen orientation device, adjusted in position and locked tightly. The section thickness was set to 4 µm. After cutting, each individual section was transferred onto a drop of distilled water on a glass slide and allowed to fully expand. The sections can be checked for proper expansion under a dissection microscope before the slide was left on the hot plate to dry and for the sections to adhere to the slide.

#### 2.4.6 Staining

After the sections dried, they were stained with a mixture of aqueous 1% (w/v) methylene blue (Gurr®) and 1% (w/v) Azure II (Gurr®) microscopy stains using the following procedure. The cold tap water was run smoothly, slowly and softly and methylene blue and

Azure II stain solution was applied directly to a slide with the sections using a dropper. After 15 seconds, the stain solution was rinsed from the slide under running tap water. Then sections were allowed to air dry overnight. The primary tissue was stained dark navy blue and secondary tissue appeared light turquoise green.

#### **2.4.7 Coverslip mounting**

To cover the sections, DePex mounting medium (Gurr®, BDH Laboratory Supplies, Poole, England) was transferred to a small vial. Then it was applied gently (to prevent air bubbles) onto the slide over the sections by using a glass rod. The sections were covered with a coverslip lowered from one side to the other. The coverslip was pressed carefully using a pencil to allow the DePex mounting medium to spread out around the coverslip edges preventing air bubbles from forming. After a few days when the DePex mounting medium was set, the DePex mounting medium outside the coverslip was trimmed away using a razor blade.

#### **2.4.8 Light microscopic observations**

The developmental sequence of *C. annuum* L. somatic embryogenesis was investigated under a light microscope.

### **2.5 Acclimatisation of somatic embryo-derived plantlets**

Plantlets derived from *C. annuum* L. somatic embryogenesis on the plantlet development medium were transferred to small pots with potting mix (40% peat, 20% sand and 20% soil) which had been autoclaved at 121°C and 15 *psi* for 20 minutes. The plantlets were covered with a transparent plastic container to maintain the humidity to 100% and were kept in a growth room at 24 °C under 16 hours illumination provided by white fluorescent lamps. After they developed 6-8 leaves and were 10-12 cm high, they were moved into bigger pots and eventually to the glasshouse if they were strong enough (also see Appendix 2).

## 2.6 *In vitro* flowering of *C. annuum* L. cv. Sweet Banana

### 2.6.1 Flower formation

The germinated somatic embryos or converted healthy plantlets were transferred to the plantlet development medium (45 ml medium in each polycarbonate tissue culture container of 8.5 cm high X 6.5 cm diameter or 50 ml medium in each container of 15 cm high X 7 cm diameter). There was one plantlet in a tissue culture container. Usually the green tiny floral bud appeared around 1.5-2 months after transfer of the plantlets to the plantlet development medium. At least 30 *in vitro* floral buds were investigated.

The vented autoclavable plant tissue culture containers (Phytakon™) were obtained from Sigma Chemical Co., St. Louis, USA. There was one plantlet in each Phytakon™ container (11 cm high X 11.5 cm diameter) with 125 ml agar-gelled plantlet development medium. At least 30 *in vitro* flowers from plantlets regenerated from immature zygotic embryos were investigated.

### 2.6.2 A comparison between *in vitro* and *in vivo* flowering of *C. annuum* L. cv. Sweet Banana

All the studies in this experiment were carried out on flowers produced by the plants in the glasshouse and those by plantlets which were regenerated via the somatic embryogenesis and then grown on the plantlet development medium under *in vitro* conditions. All the flowers were harvested on the day of their opening.

#### 2.6.2.1 Flower characters

At least 20 *in vitro* and 50 *in vivo* flowers were examined immediately after harvesting. The following parameters were studied: flower shape, calyx colour, corolla number, corolla colour, corolla length, stamen number, anther colour, anther length, filament colour, filament length, carpel number, carpel colour, carpel length, style colour, style length and stigma colour.



#### **2.6.2.2 Pollen morphology**

Stamens of a flower (usually 5-6 stamens per flower) were excised and placed on a glass slide until the anthers dehisced and released most of their pollen grains. Then all the stamens were discarded and the pollen were mounted on stubs covered with carbon conductive tabs for coating without intermediate drying. The stubs were placed straight into a diode sputter coater (SEM coating unit E5000, Polaron equipment limited, England). All specimens were coated with gold paledium for 5 minutes at high tension of 1.2 kV at 50 mA to deposit 500 nm of gold. The stubs were removed from the coater and were examined immediately using a scanning electron microscope (Leica S440, Cambridge Instruments Ltd, Cambridge, England). SEM operation was manipulated using secondary electrons at 50 pA at 15 kV. After this both *in vitro* and *in vivo* pollen grains were observed and the chosen images were recorded in the *tif* file format. Pollen size was measured from 6 different fields of observation and at least 60 pollen grains from at least 3 *in vitro* or *in vivo* flowers from different plants were examined.

#### **2.6.2.3 Pollen viability**

Pollen grains (collected as described in 2.6.2.2) of at least six flowers from different plants were mixed with 2 drops of Alexander's stain (Alexander, 1969; also see Appendix 7 for details) on a glass slide, mounted with a coverslip, and warmed over a Bunsen burner's flame for a few seconds. After this the pollen grains were examined under a light microscope. The aborted pollen grains stained green while non-aborted ones red to deep red. At least 10 fields of observation in each slide were examined.

#### **2.6.2.4 Pollen germination**

The medium of Mercado et al. (1994) comprising 0.1 mM boric acid, 1 mM calcium chloride di-hydrate, 5% (w/v) sucrose, 0.8% (w/v) agar was used for both *in vitro* and *in vivo* pollen germination test. It was autoclaved at 121°C and 15 *psi* for 20 minutes, before being dispensed into plastic Petri dishes (80 mm diameter). Pollen grains of at least six flowers from different plants were collected as described in 2.6.2.2 and were dusted uniformly over the surface of the medium in an agar plate. All the Petri dishes were

wrapped in polyethylene film and incubated at 26 °C in a dark room. Germination of pollen from both *in vitro* and *in vivo* flowers were examined under a light microscope over a period of 24 hours. At least 10 fields in each Petri dish were examined to count pollen grains with or without pollen tubes.

## **2.7 *In vitro* fruiting of *C. annuum* L. cv. Sweet Banana**

### **2.7.1 Fruit setting and ripening**

Silver nitrate ( $\text{AgNO}_3$ ) solution was added directly to the surface of the agar-gelled plantlet development medium in Phytakon<sup>TM</sup> containers. The solution (15 ml) was added to a container when tiny floral buds were formed on the plantlets. This solution was prepared in 2 ways: half MS basal medium supplemented with 20  $\mu\text{M}$   $\text{AgNO}_3$  with or without 3% (w/v) sucrose. At least 20 tiny *in vitro* fruits were investigated.

A modified plantlet development medium containing silver thiosulfate ( $\text{Ag}_2\text{S}_2\text{O}_3$ ) and sucrose was also trialled and prepared as follows. Half MS basal medium was supplemented with 5  $\mu\text{g/ml}$   $\text{Ag}_2\text{S}_2\text{O}_3$ , 1 mg/l NAA and 3% (w/v) sucrose. The liquid medium (15 ml) was added into each Phytakon<sup>TM</sup> container when tiny floral buds were formed on the plantlets. Every 2 or 3 days it was replaced with the same but freshly prepared solution. At least three fully developed abnormal fruits formed were investigated.

### **2.7.2 *In vitro* seed setting and germination**

After anthesis, the *in vitro* flowers were cut aseptically in a laminar flow cabinet. The anthers were excised and then kept in a sterile plastic Petri dish (9 cm diameter) to collect pollen which was stored in a fridge at 4 °C. As soon as other *in vitro* flowers were fully open, the pollen stored 4 °C was used to pollinate them using a sterile cotton tip. At least 6 *in vitro* fruits formed were investigated.

After the *in vitro* fruits appeared fully mature (i.e. turning completely red) at about 65 days, they were cut open for seed harvesting. The weights of the seeds before and after air drying for 6 days from the fruits were determined. Then the seeds with or without air drying were placed on a layer of Whatman No.1 filter paper moistened with 6 ml of

distilled water in a plastic Petri dish (9 cm diameter) for germination test in a dark room at 26 °C for 7 days.

## **2.8 Clonal propagation of the plantlets of *C. annuum* L. cv. Sweet Banana regenerated via somatic embryogenesis**

### **2.8.1 Sources of explants**

Nodal segments (6-8 mm long) and shoot tips (8-10 mm long) of the plantlets of *C. annuum* L. cv. Sweet Banana regenerated from somatic embryogenesis and maintained on the plantlet development medium were excised and used as explants unless indicated otherwise.

### **2.8.2 Preliminary experiments**

A published protocol (Husain et al., 1999) using MS salts and vitamin mixture (Murashige and Skoog, 1962) supplemented with 5 mg/l BA, 2 mg/l phenylacetic acid (PAA) and 3% (w/v) sucrose was trialled without modifications. Then other media trialled include MS basal medium alone or supplemented with 3% (w/v) sucrose and either 5 or 10 mg/l GA<sub>3</sub>. All the cultures were kept in a growth room at 22 °C under continuous illumination (average 26.5  $\mu\text{E}/\text{sec}/\text{m}^2$ , measured by 21X Micrologger, Campbell Scientific Inc., USA) provided by white fluorescent lamps.

### **2.8.3 Effect of growth regulator-free medium supplemented with different sucrose concentrations**

Nodal segments (15 explants) and shoot tips (8 explants) were cut and left for 6 weeks on MS basal medium containing one of the following sucrose concentrations, w/v: 0, 3 or 5%. Then, the explants were transferred to MS basal medium supplemented with either 3 or 5% (w/v) sucrose for 8 weeks. This experiment was repeated at least 3 times. All the cultures on other media were kept in a growth room at 22 °C under dim light (average 6.47  $\mu\text{E}/\text{sec}/\text{m}^2$ , measured by 21X Micrologger, Campbell Scientific Inc., USA) provided by white fluorescent lamps.

## **2.9 Statistical analysis and data handling**

Means and standard error of the means were first determined. Then the data were subjected to analysis of variance to find and if required, Tukey (HSD) comparison of means was performed using “Statistix for Windows version 7.0” (Analytical Software, USA). All the figures were created using “Microsoft® Excel 97” and “Microsoft® Word 97” (Microsoft Corporation, USA).

## Chapter 3

# Results and Discussion

### 3.1 Evaluation of *Capsicum annuum* L. somatic embryogenesis protocols

There have been many experiments on *in vitro* organogenesis of pepper (e.g., Fari, 1986; Morrison et al., 1986; Ramage and Leung, 1996; Steinitz et al., 1999). In comparison, only a few reports on *Capsicum* somatic embryo formation have appeared in the last decade (immature zygotic embryo as explant: Harini and Lakshmi Sita, 1993; Jeong et al., 1994; Binzel et al., 1996; Jo et al., 1996; mature zygotic embryo as explant: Buyukalaca and Mavituna, 1996; leaves of mature plants as explant: Kintzios et al., 1998; Kintzios et al., 2000; Kintzios et al., 2001). Of these protocols, that using mature embryo as explant appears to be the most attractive and convenient as there is no need to grow plants to the appropriate developmental stages in the glasshouse. In this study the question of whether this mature zygotic embryo method is also applicable to pepper cultivars other than Ace was first explored. In addition, some aspects of the other published protocols using immature zygotic embryo explants for somatic embryogenesis in *Capsicum* were also investigated.

#### 3.1.1 Mature zygotic embryo as explant [the procedure of Buyukalaca and Mavituna (1996)]

The mature zygotic embryos of *Capsicum annuum* L. cvs. Sweet Banana, California Wonder and Yolo Wonder developed callus well at the somatic embryo initiation step on MS basal medium supplemented with 2 mg/l 2,4-D and 3% (w/v) sucrose. This suggests

that the cv. Ace and these 3 cultivars responded similarly on the embryogenic callus induction medium described by Buyukalaca and Mavituna (1996).

In the cv. Ace, the white-watery callus was nonembryogenic while the yellowish-nodular-friable callus was highly embryogenic (Buyukalaca and Mavituna, 1996). In comparison, it would seem that cvs. California Wonder (Plate 3.1 a, b and c) and Yolo Wonder had formed what appeared to be embryogenic callus but cv. Sweet Banana had not (Table 3.1). Hence, only the callus initiated from the former 2 cultivars was investigated further.

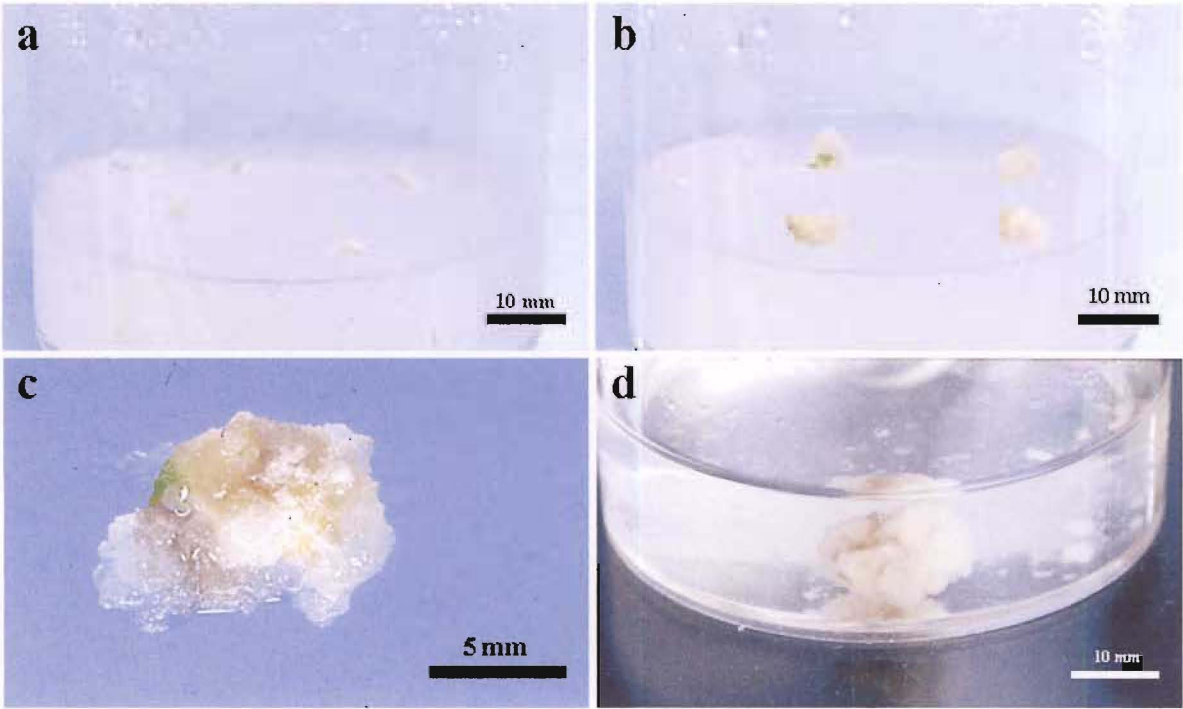
**Table 3.1** Appearance of callus formed by mature zygotic embryos of 3 cultivars of *Capsicum annuum* L after 4 weeks of culture.

Cultivar	Callus morphology
Sweet Banana	White-watery
California Wonder	Yellowish-friable
Yolo Wonder	Yellowish-friable

Upon transfer to liquid MS basal medium supplemented with 1 mg/l 2,4-D and 3% (w/v) sucrose, the yellowish-friable callus of cvs. California Wonder (Plate 3.1 d) and Yolo Wonder initiated cell suspensions which grew well. However, the cell suspensions failed to develop into somatic embryos after passing through the process of pretreatment in liquid MS basal medium (KNO<sub>3</sub> free) supplemented with 6 g/l potassium citrate, 2 mg/l 2,4-D and 3% (w/v) sucrose for 3 weeks and embryo initiation in liquid MS basal medium supplemented with 6 g/l L-proline, 10 mM NH<sub>4</sub>NO<sub>3</sub> and 3% (w/v) sucrose for 3 weeks as described by Buyukalaca and Mavituna (1996). This suggests that the success of this procedure is dependent on cultivars and it may only work with the cv. Ace. However, in my hands after several attempts, even with the mature embryos of cv. Ace as explants and

**Plate 3.1** Induction of callus formation in mature zygotic embryo explant of *Capsicum annuum* L. cv. California Wonder using the protocol of Buyukalaca and Mavituna (1996)

- (a) excised mature zygotic embryo explants
- (b) callus formed after 4 weeks of culture
- (c) a close-up view of the yellowish-friable callus formed
- (d) initiation of cell suspension culture starting with the yellowish-friable callus





following faithfully the original published procedure, no somatic embryos were formed. The reason for this is not known at present.

### **3.1.2 Immature zygotic embryo as explants**

Pepper somatic embryos were first obtained starting from zygotic embryos isolated from immature seeds of *C. annuum* L. cv. California Wonder (Harini and Lakshmi Sita, 1993). The present study showed that immature zygotic embryos of 3 additional cultivars (Sweet Banana, Yolo Wonder and Ace) could also develop somatic embryo structures using the embryo initiation and maturation medium of Harini and Lakshmi Sita (1993). However, the percentage of somatic embryo structure formed (Plate 3.2b) was very low in all 3 cultivars. The number of plantlets resulted after transfer of the somatic embryo structures to the germination or conversion medium as described in the protocol of Harini and Lakshmi Sita (1993) was also very low. The main problem seems to be the failure of most somatic embryo structures to convert into complete plantlets while most of these structures developed friable or compact callus instead. Besides, most of the resulting plantlets appeared to be abnormal in the following respects: they did not have cotyledon-like or leaf-like structure and their stems appeared to be twisted (compare Plate 3.2c with 3.2d).

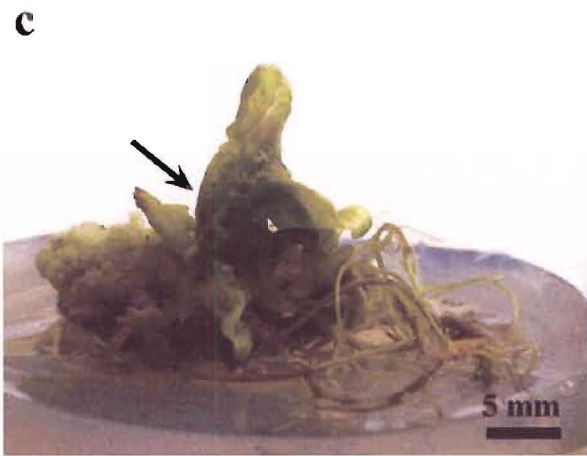
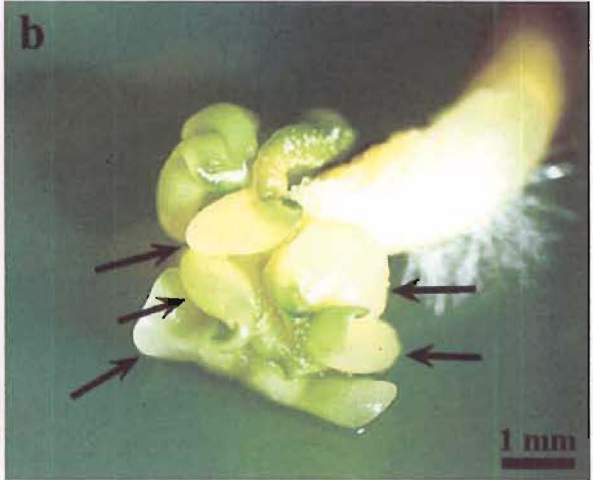
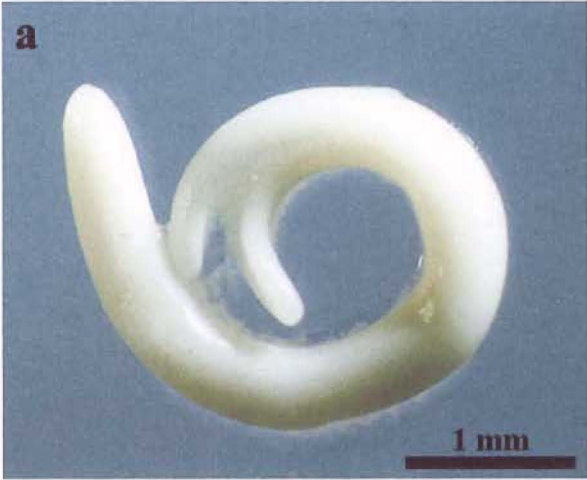
## **3.2 Critical factors influencing somatic embryogenesis from immature zygotic embryo explants**

### **3.2.1 Size of immature zygotic embryo explants**

In a preliminary study, it was confirmed that the size of immature zygotic embryo explants was one of the important factors for pepper somatic embryogenesis as suggested in the previously published protocols and the appropriate embryo size was different among cultivars as described by Harini and Lakshmi Sita (1993) and Binzel et al. (1996). Of the 4 cultivars used here, it was found that 5-7 mm immature zygotic embryo explant gave the best somatic embryogenesis response. However, other characteristics of the immature seed also appeared to be important. For example, if the immature zygotic embryo explants appeared transparent, no somatic embryo structure was formed even if it was of the appropriate size. Also, the explant should not be of U-shape but the cotyledons should curl

**Plate 3.2** Somatic embryogenesis in *Capsicum annuum* L. cv. Sweet Banana.

- (a) a typical immature zygotic embryo explant from glasshouse-grown plants
- (b) well-formed somatic embryo structures (arrows) on the embryonic axis and cotyledons of the immature zygotic embryo explant after 3 weeks of culture
- (c) appearance of an abnormal plantlet (arrow)
- (d) appearance of a normal plantlet



toward the embryonic axis (Plate 3.2a). Those immature zygotic embryo explants isolated from seeds with fully-developed cream-like endosperm which was still soft were more likely to form somatic embryos.

### **3.2.2 Time on initiation and maturation medium**

According to the procedures of Harini and Lakshmi Sita (1993) and Binzel et al. (1996), immature zygotic embryo explants were cultured for 42 and 24 days, respectively, in order to obtain somatic embryos and eventual plantlet conversion. However, in a preliminary experiment it was found that besides a low percentage of somatic embryo induction resulted, abundant callus was also formed. In this research aiming to improve somatic embryogenesis in peppers, the initiation and maturation medium of Harini and Lakshmi Sita (1993) was modified by not adding any coconut water and herein called induction medium.

If the immature zygotic embryo explants of Sweet Banana and California Wonder peppers were left on induction medium for only 1 week, no somatic embryo structures were formed. Instead, these explants germinated and grew into normal seedlings. Unlike somatic embryogenesis in peanut, the optimum induction period was found to be 7 days (Baker and Wetzstein, 1998).

It was found that somatic embryo formation could be induced and converted into plantlets after the immature zygotic embryo explants had been cultured on the induction medium for as little as 2 weeks before they were transferred to the germination or conversion medium (Table 3.2). In fact, somatic embryo structures could be first observed after 12-14 days (sometimes 10 days) on the induction medium. The more time immature zygotic embryo explants were left on the induction medium, the more visible these somatic embryo structures became. The data (Table 3.2) suggest that the percentage of somatic embryo induction was not significantly different between 2 and 4 weeks of incubation on the induction medium. In contrast, the percentage of plantlet conversion notably decreased if the explants with the somatic embryo structures were left longer than 2 weeks on the induction medium. It is likely that during the first 2 weeks 2,4-D may stimulate the formation of somatic embryo structures but with longer exposure to 2,4-D their subsequent development into complete plantlets could be inhibited.

**Table 3.2** Influence of exposure duration (weeks) to somatic embryo induction medium on somatic embryogenesis in immature zygotic embryo explants of *Capsicum annuum* L. cvs. Sweet Banana and California Wonder.

Cultivars	weeks	Number of explants*	% Induction*	% Conversion*	SE number*	% Healthy plants*
Sweet Banana	2	82	78.8±6.9a	75.0±7.2a	3.1±0.4a	6.7±1.1a
	4	66	69.9±11.8a	39.8±10.1b	2.0±0.2b	2.0±0.2a
California	2	120	83.2±6.0a	64.6±8.4a	2.8±0.2a	6.0±0.4a
Wonder	4	89	86.8±4.9a	28.3±6.2b	1.5±0.4b	2.4±0.1b

\*Values shown are means± S.E. of data obtained from 6 and 7 replicate experiments in each treatment in the case of Sweet Banana and California Wonder, respectively. Data from a cultivar marked by same letter in a column are not significantly different (ANOVA,  $P < 0.05$ ). For each treatment, number of explants = immature zygotic embryos used in all the replicate experiments; % induction = the percentage of immature zygotic embryo explants that formed somatic embryo structures; % conversion = the percentage of immature zygotic embryo explants that formed plantlets (whether they appeared normal or not); SE number = number of somatic embryo structures forming per explant; % Healthy plants = the percentage of plantlets which appeared normal.

### 3.2.3 Requirement for coconut water

Coconut water, a source of cytokinin among other potentially beneficial factors, was claimed to be more effective for induction of pepper somatic embryo according to the procedures of Harini and Lakshmi Sita (1993) and Binzel et al. (1996). However, in the protocol of Jo et al. (1996) working on a different pepper cultivar, coconut water was not included in the somatic embryo induction medium. In the present research, the data showed that somatic embryo structures could be formed equally well in all 3 cultivars (Sweet Banana, California Wonder and Ace) if not better on medium without coconut water (Table 3.3). The percentage of plantlet conversion was not significantly different between the 2 treatments in the case of cvs. Sweet Banana and Ace but different in the case of cv. California Wonder. In cv. Yolo Wonder, the experiment had been done only on medium without coconut water and comparable high percentages of induction and conversion were obtained as in the same experiment with the other 3 cultivars (data not shown).

### 3.2.4 Effect of light and dark condition at the induction stage on pepper somatic embryogenesis

Light or dark condition could be an important factor for somatic embryo induction. For example, somatic embryogenesis of garden leek (*Allium porrum* L.) has a requirement for light (Hong and Debergh, 1995) but that of olive and Camellia could occur in complete darkness (San-Jose and Vieitez, 1993; Rugini and Caricato, 1995). This requirement for pepper somatic embryogenesis was examined in the present study.

Immature zygotic embryo explants from 3 cultivars, Sweet Banana, California Wonder and Yolo Wonder, were kept under continuous illumination or in the dark for about 2 weeks. Then, all the explants were transferred to the germination or conversion medium and kept under continuous illumination. It was found that induction of somatic embryo structures of all 3 cultivars could occur equally well in the light or dark condition (Table 3.4). Moreover, plantlet conversion seems to be independent of the prior induction in the light or dark condition. In the red pepper (*C. annuum* L. cv. Nokkwang), somatic embryos were induced in the dark within 3 weeks (Jo et al., 1996), but the effect of light was apparently not studied.

**Table 3.3** Influence of somatic embryo induction medium supplemented with or without coconut water on somatic embryogenesis in immature zygotic embryo explants of *Capsicum annuum* L. cvs. Sweet Banana, California Wonder and Ace.

Cultivars	Coconut water	Number of explants*	% Induction*	% Conversion*	SE number*	% Healthy plants*
Sweet Banana	With	290	64.2±4.0a	61.5±3.8a	2.7±0.2a	13.3±0.9a
	Without	270	70.3±4.0a	64.0±4.2a	2.6±0.2a	6.0±0.4a
California	With	75	42.1±10.6a	25.8±8.6a	1.6±0.4a	6.5±0.3a
Wonder	Without	292	77.7±4.2b	55.2±5.7b	2.6±0.2b	3.7±0.3a
Ace	With	56	24.5±4.7a	20.8±4.9a	1.4±0.2a	0
	Without	55	32.5±5.1a	22.1±9.1a	1.5±0.4a	0

\*Values shown are means± S.E. of data obtained from 22 and 4 replicate experiments in each treatment in the case of Sweet Banana and Ace, respectively; 5 and 16 of replicate experiments in the case of California Wonder with or without coconut water, respectively. Data from a cultivar marked by same letter in a column are not significantly different (ANOVA,  $P < 0.05$ ). For each treatment, number of explants = immature zygotic embryos used in all the replicate experiments; % induction = the percentage of immature zygotic embryo explants that formed somatic embryo structures; % conversion = the percentage of immature zygotic embryo explants that formed plantlets (whether they appeared normal or not); SE number = number of somatic embryo structures forming per explant; % Healthy plants = the percentage of plantlets which appeared normal.

**Table 3.4** Effect of incubating immature zygotic embryo explants under continuous illumination or in the dark for 2 weeks on somatic embryogenesis of *Capsicum annuum* L. cvs. Sweet Banana, California Wonder and Yolo Wonder.

Cultivars	Treatment	Number of explants*	% Induction*	% Conversion*	SE number*	% Healthy plants*
Sweet Banana	Light	61	54.8±6.8a	49.1±8.5a	1.9±0.2a	4.1±0.4a
	Dark	71	67.1±9.4a	67.1±9.4a	2.3±0.5a	13.0±1.6a
California Wonder	Light	71	68.1±6.4a	55.0±11.2a	2.0±0.3a	0
	Dark	72	60.8±4.4a	44.2±7.4a	1.6±0.2a	0
Yolo Wonder	Light	64	51.4±6.9a	31.2±8.7a	2.1±0.5a	2.0±0.2a
	Dark	66	51.7±6.2a	36.8±11.1a	1.7±0.3a	2.0±0.2a

\*Values shown are means± S.E. of data obtained from 5 replicate experiments in each treatment of the different cultivars. Data from a cultivar marked by same letter in a column are not significantly different (ANOVA,  $P < 0.05$ ). For each treatment, number of explants = immature zygotic embryos used in all the replicate experiments; % induction = the percentage of immature zygotic embryo explants that formed somatic embryo structures; % conversion = the percentage of immature zygotic embryo explants that formed plantlets (whether they appeared normal or not); SE number = number of somatic embryo structures forming per explant; % Healthy plants = the percentage of plantlets which appeared normal.



### 3.2.5 Effect of sugar type at the induction stage on pepper somatic embryogenesis

Sugar, a carbon and energy source, could be a critical factor for somatic embryogenesis. Different plant species may need different types of sugars for somatic embryogenesis. In *Panax ginseng*, the best regeneration of somatic embryos was obtained on medium containing glucose (Tang, 2000) while somatic embryo production of *Hevea brasiliensis* was significantly higher on a maltose-containing medium (Blanc et al., 1999). This requirement for pepper somatic embryogenesis was examined in the present study using the cv. Yolo Wonder. Immature zygotic embryo explants of this cultivar were placed on the induction medium with or without different types of sugars for about 2 weeks. Then, they were transferred to the germination or conversion medium. It was found that somatic embryo structures could develop only on the medium containing sucrose or maltose (Table 3.5). On media supplemented without sugar or with glucose or fructose, the explants initially turned red and exhibited no further development (data not shown). The effect of sucrose and maltose were not significantly different (Table 3.5). However, no healthy plantlet was recovered after culture on the maltose-containing medium (data not shown). The present finding is different from other reports showing a promotive effect of maltose in a number of embryogenesis systems, for example, asparagus somatic embryo development (Kunitake et al., 1997) and pollen embryogenesis in bell peppers (Dolcet-Sanjuan et al., 1997). It appears worthwhile for future studies examining more closely the effect of maltose and other carbohydrates in somatic embryogenesis of different pepper cultivars.

### 3.2.6 Replacement of coconut water with BA

Kintzios et al. (2000) showed that somatic embryogenesis in pepper leaves was induced using 2.9 mg/l BA. Here, it was found that on induction medium without coconut water but with 2.9 mg/l BA the immature zygotic embryos of the 3 cultivars (Sweet Banana, California Wonder and Yolo Wonder) could not develop somatic embryo structures (Table 3.6). The whole explants became swollen and the cotyledons turned green. After transfer to the germination or conversion medium, they all formed callus of different appearances. In cv. 'Sweet Banana', the callus formed was of the white-watery and light green compact types, whereas in cvs. 'California Wonder' and 'Yolo Wonder' green or yellowish

**Table 3.5** Effect of 10% (w/v) maltose or sucrose in the somatic embryo induction medium on somatic embryogenesis in immature zygotic embryo explants of *Capsicum annuum* L. cv. Yolo Wonder.

Treatment	Number of explants*	% Induction*	% Conversion*	SE number*	% Healthy plants*
Maltose	34	59.4±11.2a	51.4±13.8a	2.0±0.3a	0
Sucrose	44	77.1±6.5a	53.6±15.3a	2.3±0.6a	9.3±0.7

\*Values shown are means± S.E. of data obtained from 4 replicate experiments in each treatment. Data marked by same letter in a column are not significantly different (ANOVA,  $P < 0.05$ ). For each treatment, number of explants = immature zygotic embryos used in all the replicate experiments; % induction = the percentage of immature zygotic embryo explants that formed somatic embryo structures; % conversion = the percentage of immature zygotic embryo explants that formed plantlets (whether they appeared normal or not); SE number = number of somatic embryo structures forming per explant; % Healthy plants = the percentage of plantlets which appeared normal.

**Table 3.6** Effect of 2.9 mg/l BA in the somatic embryo induction medium on somatic embryogenesis in immature zygotic embryo explants of *Capsicum annuum* L. cvs. Sweet Banana, California Wonder and Yolo Wonder.

Cultivars	Treatment	Number of explants*	% Induction*	% Conversion*	SE number*	% Healthy plants*
Sweet Banana	With BA	48	0	0	0a	0a
	Without BA	48	78.9±8.1	67.8±10.0	2.7±0.2b	8.3±0.7b
California Wonder	With BA	45	0	0	0a	0a
	Without BA	45	93.8±6.3	79.9±7.9	3.2±0.4b	5.3±0.5b
Yolo Wonder	With BA	45	0	0	0a	0a
	Without BA	50	72.2±8.5	48.9±15.6	2.3±0.6b	11.6±0.6a

\*Values shown are means± S.E. of data obtained from 4 replicate experiments in each treatment of the different cultivars. For each treatment, number of explants = immature zygotic embryos used in all the replicate experiments; % induction = the percentage of immature zygotic embryo explants that formed somatic embryo structures; % conversion = the percentage of immature zygotic embryo explants that formed plantlets (whether they appeared normal or not); SE number = number of somatic embryo structures forming per explant; % Healthy plants = the percentage of plantlets which appeared normal.

compact callus was formed. This level of BA might be inhibitory to somatic embryogenesis in the present study.

### 3.2.7 Effect of Yariv's reagent

There are some data suggesting that arabinogalactan protein (AGPs) could play a role in somatic embryogenesis (Stacey et al., 1990; Kreuger and van Host, 1993; Egertsdotter and Von Arnold, 1995; Saare et al., 2000). The main experimental approach has been the use of Yariv's reagent (Yariv et al., 1962) in culture medium and studying its effect on somatic embryogenesis in cell suspension cultures (Thompson and Knox, 1998; Chapman et al., 2000). The effect of Yariv's reagent on somatic embryogenesis in immature zygotic embryo explants of peppers or other plants have never been investigated. Here, this was studied using 2 cultivars of *Capsicum*, California Wonder and Yolo Wonder.

After the immature zygotic embryo explants of *C. annuum* L. cv. California Wonder were cultured on induction medium supplemented with 20 µg/ml of Yariv's reagent (βGluY) for 16 days, less somatic embryo structures were found on the cotyledons when compared to the control (Table 3.7; compare Plate 3.3 a with b). Somatic embryo structures developed slower on the explants after transfer to the germination or conversion medium supplemented with Yariv's reagent (compare Plate 3.3 c with d; e with f) although statistically there was no difference between the control and the Yariv's reagent-containing medium as far as conversion is concerned (Table 3.7).

This result was similar to another experiment of culturing immature zygotic embryo explants of *C. annuum* L. cv. Yolo Wonder on induction medium supplemented with Yariv's reagent for 6-7 weeks.

Thus, arabinogalactan proteins could be one of the important factors in *Capsicum* somatic embryogenesis. More in depth research in this direction is warranted.

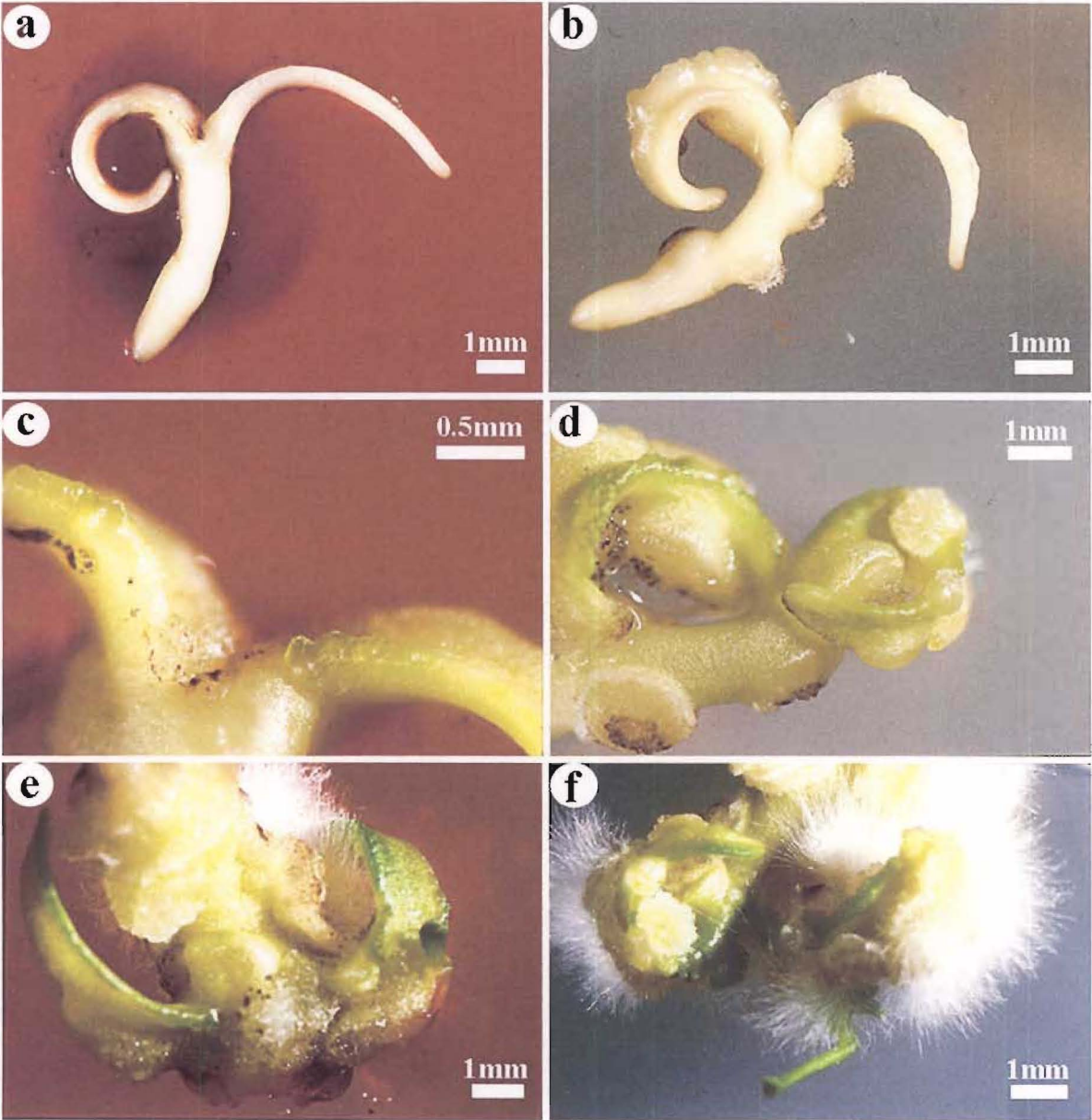
**Table 3.7** Effect of 20 µg/ml Yariv's reagent in the somatic embryo induction medium on somatic embryogenesis in immature zygotic embryo explants of *Capsicum annuum* L. cv. California Wonder.

Treatment	Number of explants*	% Induction*	% Conversion*	SE number*	% Healthy plants*
With Yariv's reagent	39	18.0±14.3a	12.8±9.3a	0.4±0.4a	0
Without Yariv's reagent	40	70.1±12.1b	42.4±16.9a	2.2±0.2b	2.0±0.7

\*Values shown are means± S.E. of data obtained from 3 replicate experiments in each treatment. Data marked by same letter in a column are not significantly different (ANOVA,  $P < 0.05$ ). For each treatment, number of explants = immature zygotic embryos used in all the replicate experiments; % induction = the percentage of immature zygotic embryo explants that formed somatic embryo structures; % conversion = the percentage of immature zygotic embryo explants that formed plantlets (whether they appeared normal or not); SE number = number of somatic embryo structures forming per explant; % Healthy plants = the percentage of plantlets which appeared normal.

**Plate 3.3** Effect of Yariv's reagent on somatic embryogenesis of *Capsicum annuum* L. cv. California Wonder.

- (a) on induction medium supplemented with Yariv's reagent for 16 days
- (b) on induction medium without Yariv's reagent for 16 days
- (c) on conversion medium supplemented with Yariv's reagent for 5 days
- (d) on conversion medium without Yariv's reagent for 5 days
- (e) on conversion medium supplemented with Yariv's reagent for 12 days
- (f) on conversion medium without Yariv's reagent for 12 days



### **3.3 Formation of putative somatic embryo structures on immature zygotic embryo explants of *C. annuum* L.**

In the present study, both morphological and histological changes showing direct somatic embryogenesis in immature zygotic embryo explants of *C. annuum* L., mainly cv. Sweet Banana, are reported.

For morphological changes, explants on the induction medium and germination or conversion medium were examined under a dissection microscope at various stages. At the early stage of culture on induction medium (day 3), the cotyledons of immature zygotic embryo explants of *C. annuum* L. cv. Sweet Banana curled toward the embryonic axis as on the day 0 (compare Plate 3.4 a and b). After 5 days on induction medium the cotyledons split further apart (Plate 3.4 c). Around 5-7 days on induction medium, somatic embryo structure was still hardly detected (Plate 3.4 c and d). Somatic embryo structures were more easily seen with the aid of a microscope after 14-16 days of culture on the induction medium (Plate 3.4 f and g). Sometime these macroscopic structures could be found as early as day 10 (Plate 3.4 e). Similar observations were obtained during culture of the immature zygotic embryo explants of cv. Yolo Wonder (Plate 3.5).

The putative somatic embryo structures turned green 5-7 days after the explants were transferred to the germination or conversion medium. After 7-10 days, they developed into plantlets.

### **3.4 Histology of immature zygotic embryo explants of *C. annuum* L. cv.**

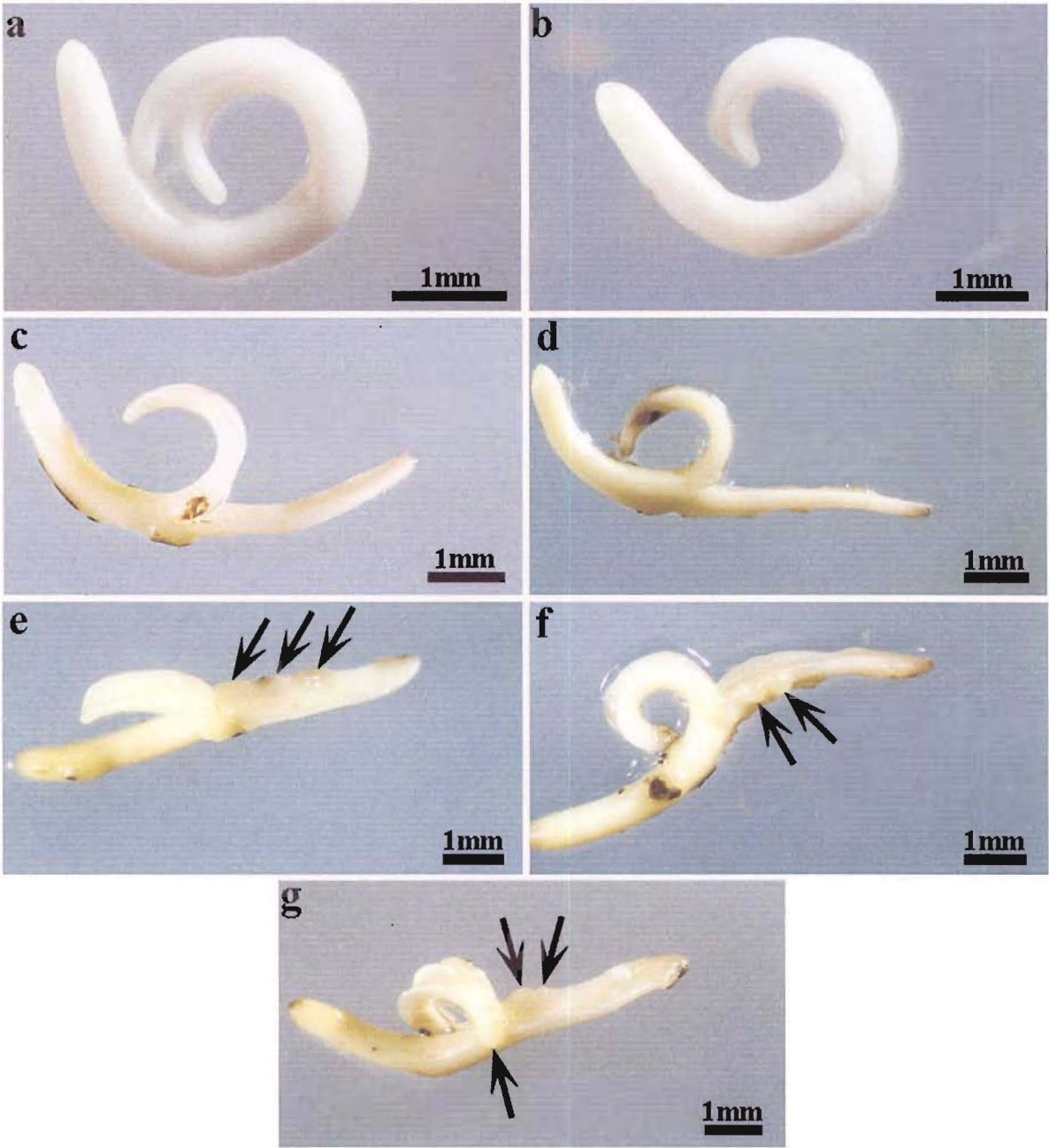
#### **Sweet Banana during somatic embryogenesis**

There are only two publications showing light microscopic images, particularly the late stages of somatic embryo development in *Capsicum* (Harini and Lakshmi Sita, 1993; Binzel et al., 1996). However, the origin and sequence of pepper somatic embryogenesis are still unclear. Here, sections from the cotyledon region of the immature zygotic embryo explants cultured on somatic embryo induction medium were prepared for light microscopy. In general, there are two main methods to prepare plant materials for light microscopy. The first one is paraffin infiltration and embedding method and another one is



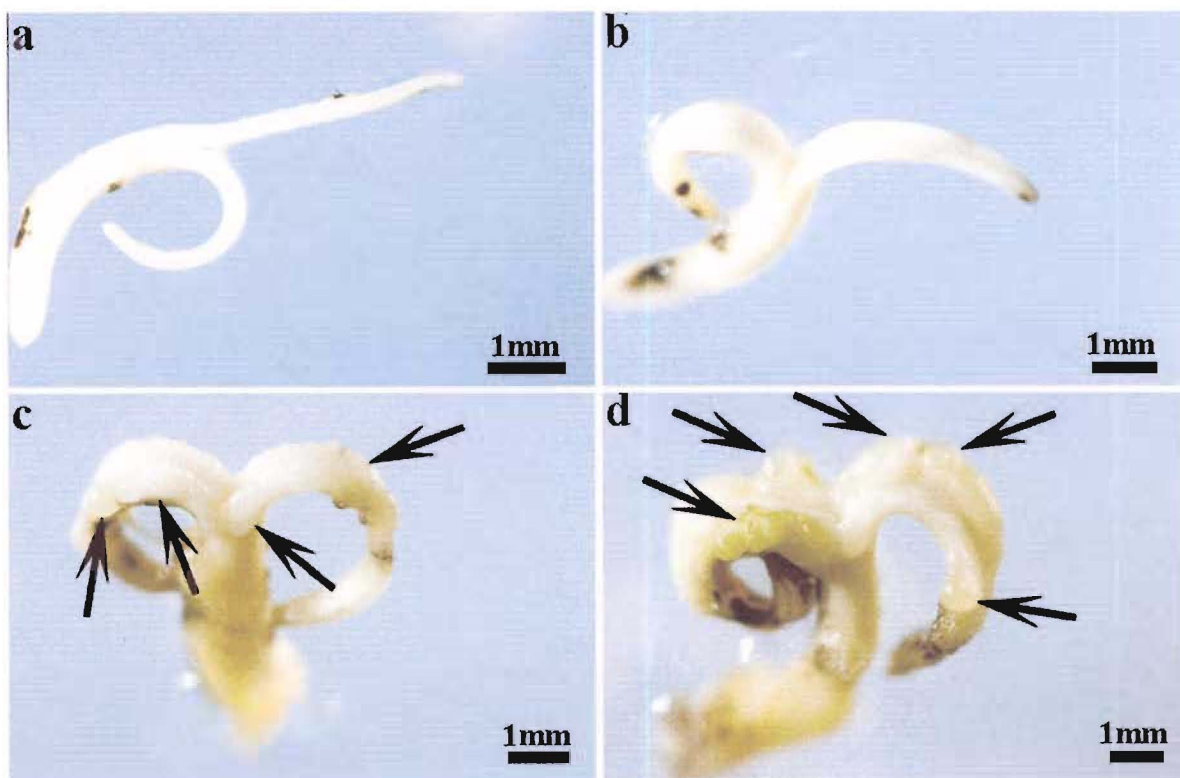
**Plate 3.4** Macroscopic changes on immature zygotic embryo explant of *Capsicum annuum* L. cv. Sweet Banana placed on somatic embryo induction medium for different times. The arrows indicate structures putatively associated with somatic embryogenesis.

- (a) day 0
- (b) day 3
- (c) day 5
- (d) day 7
- (e) day 10
- (f) day 14
- (g) day 16



**Plate 3.5** Macroscopic changes on immature zygotic embryo explant of *Capsicum annuum* L. cv. Yolo Wonder placed on somatic embryo induction medium for different times. The arrows indicate structures putatively associated with somatic embryogenesis.

- (a) day 7
- (b) day 10
- (c) day 14
- (d) day 16



a hard plastic infiltration and embedding. There are many benefits in the latter method over the former such as preventing the shrinkage of sample, making thinner sections (1  $\mu\text{m}$  or less) possible, resulting in little or no knife damage and allowing better stain accessibility (Fowke and Rennie, 1995). In the present study, resin, a hard plastic, infiltration and embedding method was used. Glutaraldehyde used in this research was a better fixative than FAA (formalin/acetic acid/alcohol) which was used in the previous histological studies (Harini and Lakshmi Sita, 1993; Binzel et al., 1996). Moreover, the resin infiltration and embedding method appears to be better than the paraffin method used in the two previous studies (Harini and Lakshmi Sita, 1993; Binzel et al., 1996). This is because greater details of cell characteristics could be observed more easily using the thin sections (3-4  $\mu\text{m}$ ) in the present study while the previous protocols used 10  $\mu\text{m}$  thick sections.

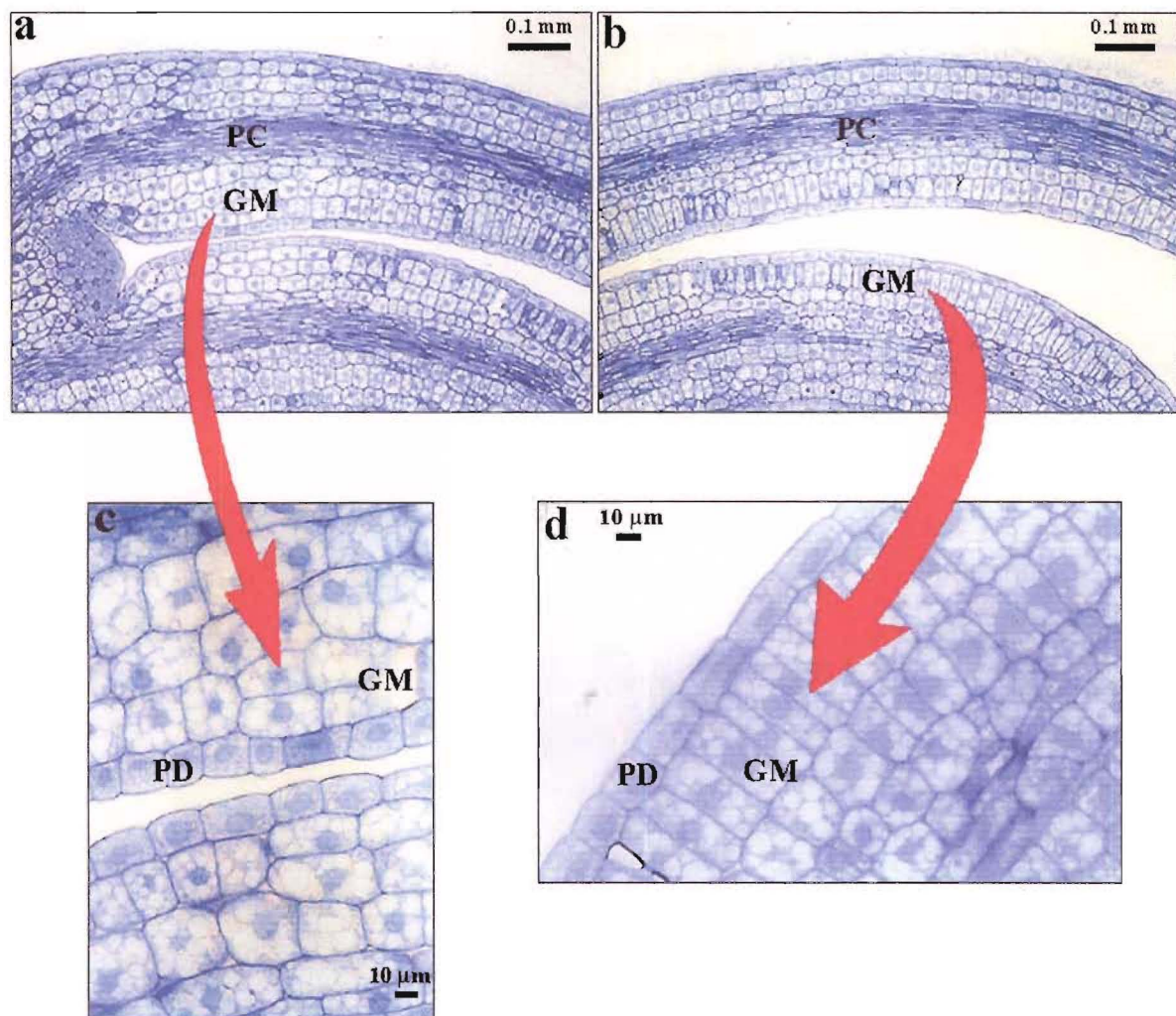
At the beginning (day 0), two main types of cell were found underneath the protoderm of an immature zygotic embryo explant: cuboidal or rectangular cells which were near the shoot apex (Plate 3.6 a and c) and columnar-shaped cells (Plate 3.6 b and d) which were farther away from the shoot apex. Overall, the cotyledons of the immature zygotic embryo explant exhibited typical anatomy of the cotyledons of dicotyledoneous plants.

At 3-5 days after of culture on induction medium, the cells underneath the protoderm in some parts of cotyledon were deviding periclinaly (Plates 3.7 and 3.8) At day 7, cell division in the same region occurred in both periclinal and anticlinal directions resulting in more cells (Plate 3.9 a and b). This pattern of cell division associated with pepper somatic embryogenesis is different from that in other plant species. For example, the irregular, periclinal and oblique quantal divisions associated with somatic embryogenesis occurred in the epidermal cells of *Trifolium repens* hypocotyl (Maheswaran and Williams, 1985), in the epidermis and subepidermis of the cotyledon explant of *Camellia japonica* L. (Barciela and Vieitez, 1993) and anticlinal division occurred in the epidermal cells of geranium hypocotyl (Hutchinson et al., 1996).

The protoderm of the cotyledon was seen protruding when the cells underneath divided more and more. The putative somatic embryo structures which were globular and lying on the inner surface of the cotyledons were evident at around 10-16 days (Plate 3.9 c-e). These structures were similar to the globular somatic embryos described previously by

**Plate 3.6** Histology of immature zygotic embryo explant of *Capsicum annuum* L. cv. Sweet Banana on induction medium at day 0.

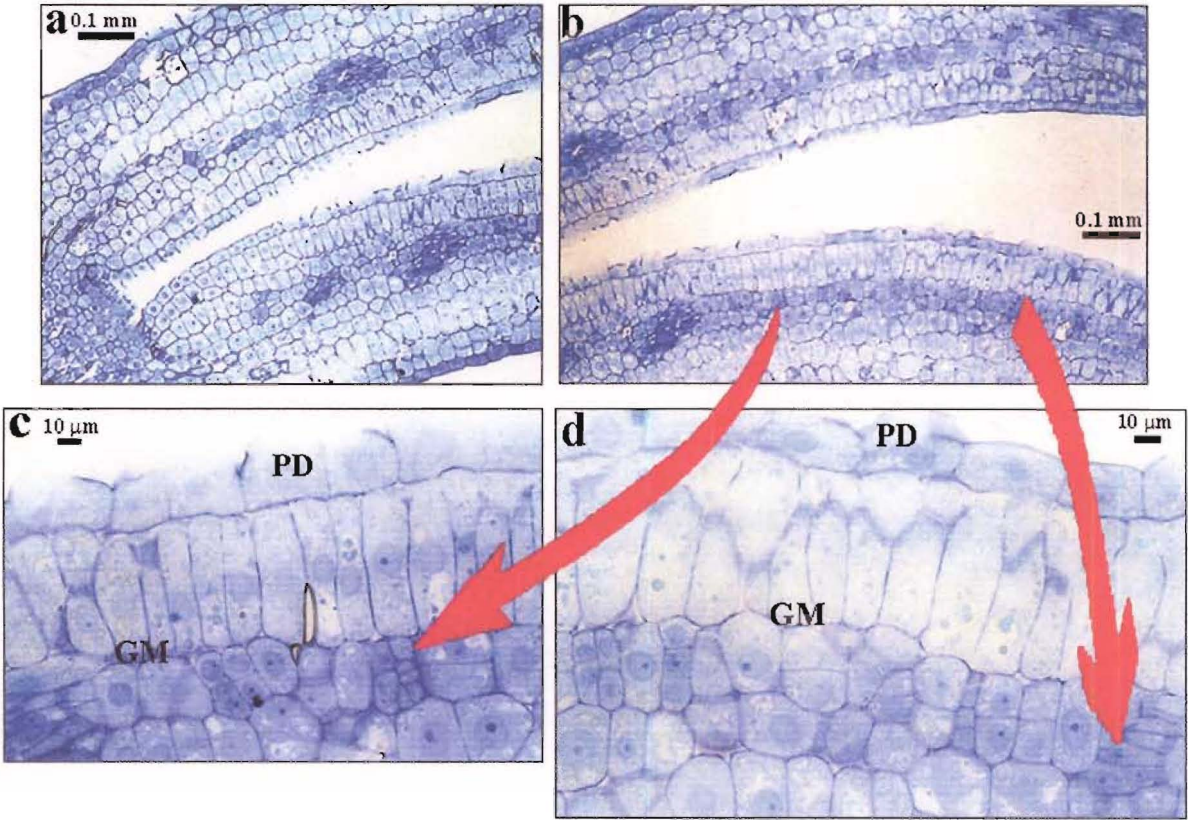
- (a) shoot apex region (GM = ground meristem; PC = procambium)
- (b) cotyledonary region farther away from the shoot apex (GM = ground meristem; PC = procambium)
- (c) cells in the ground meristem of cotyledon near the shoot apex (PD = protoderm; GM = ground meristem)
- (d) cells in the ground meristem of cotyledon farther away from the shoot apex (PD = protoderm; GM = ground meristem)



**Plate 3.7** Histology of immature zygotic embryo explant of *Capsicum annuum* L. cv. Sweet Banana on induction medium at day 3.

- (a) shoot apex region
- (b) cotyledonary region farther away from the shoot apex
- (c) and (d) dividing cells in the different part of ground meristem of the cotyledon (PD = protoderm; GM = ground meristem)



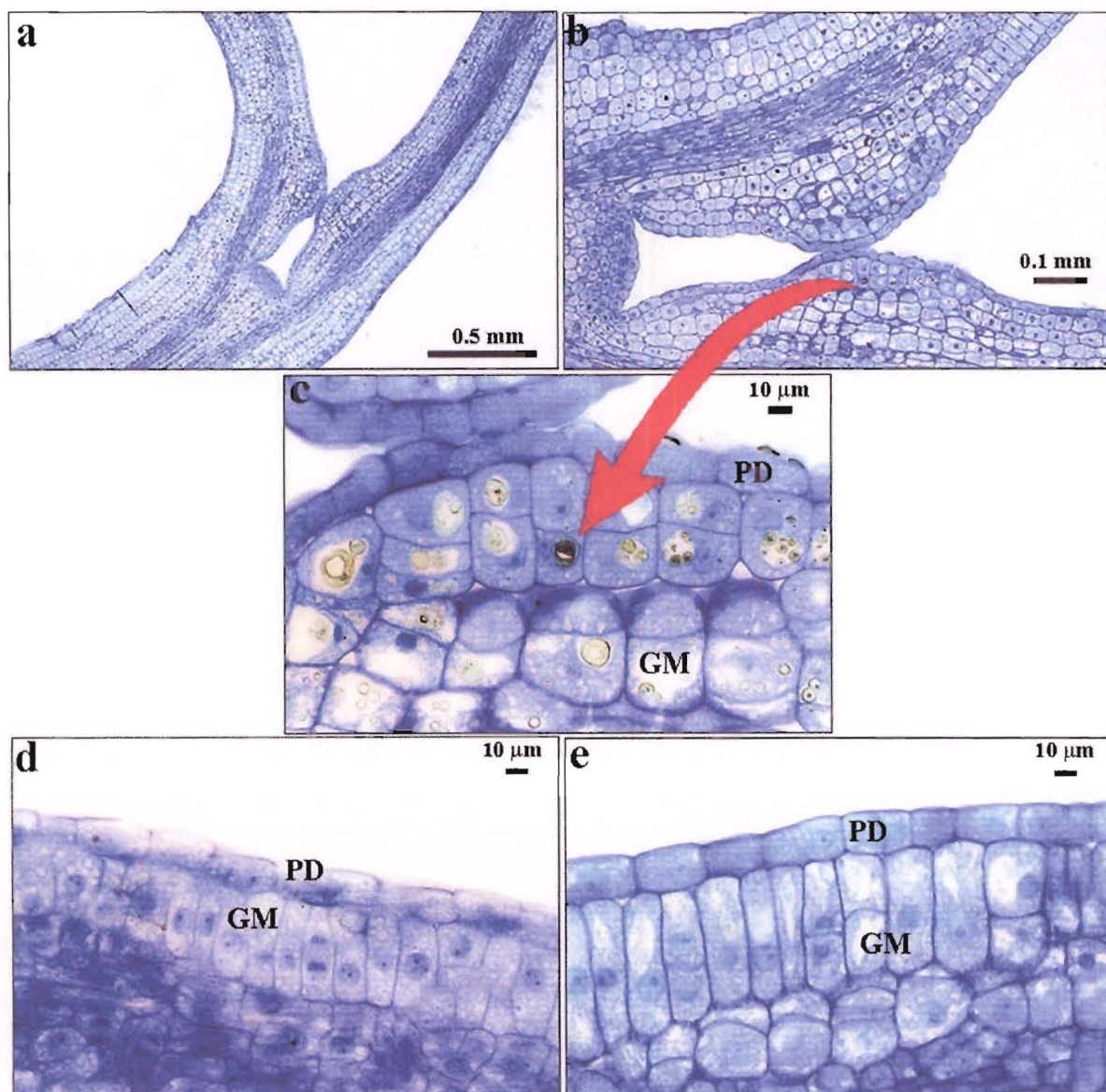


**Plate 3.8** Histology of immature zygotic embryo explant of *Capsicum annuum* L. cv. Sweet Banana on induction medium at day 5.

(a) shoot apex region

(b) somatic embryo structures developing near shoot apex

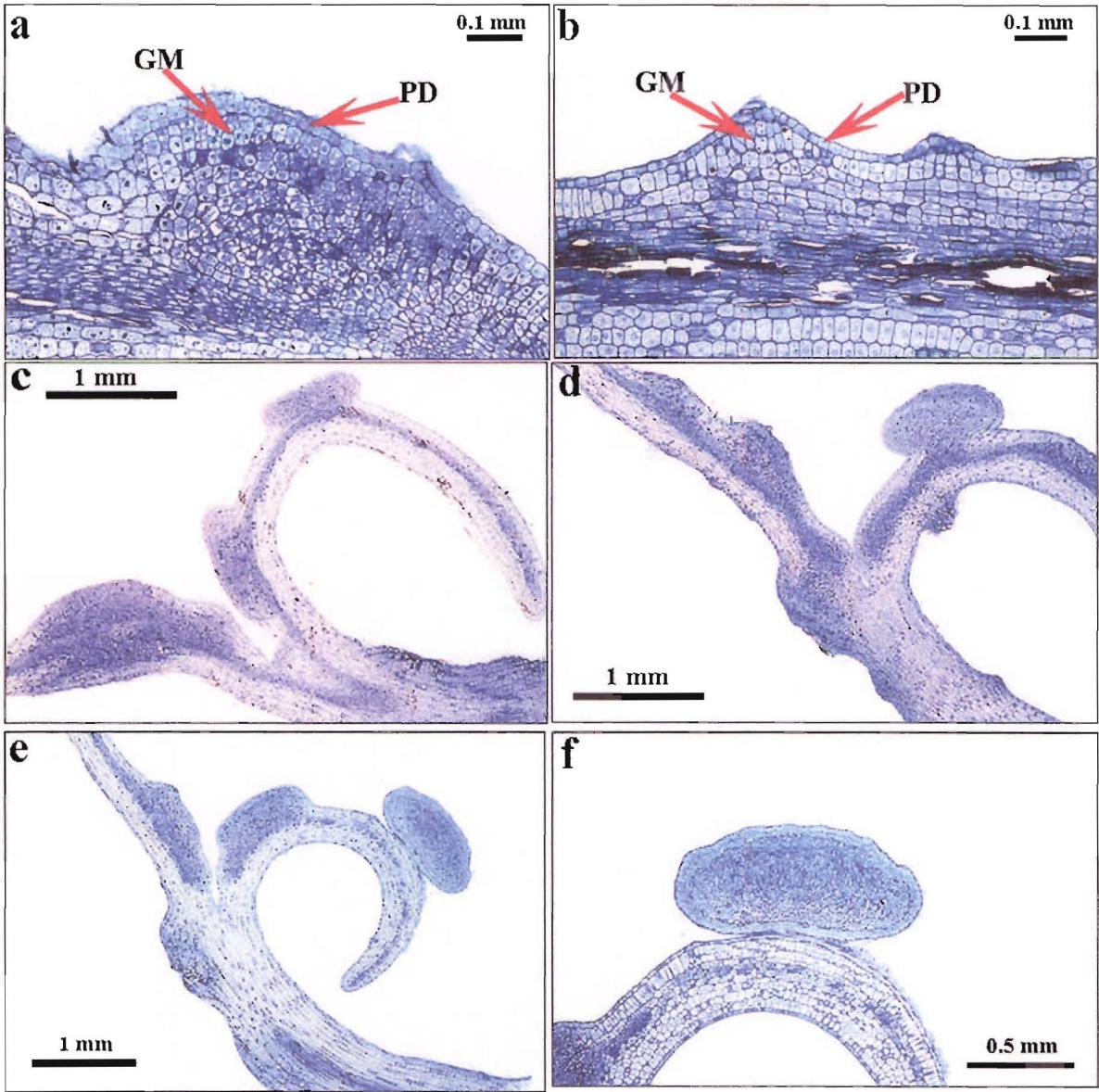
(c) (d) and (e) dividing cells in ground meristem (GM) of the cotyledon (PD = protoderm)



**Plate 3.9** Histology of immature zygotic embryo explant of *Capsicum annuum* L. cv. Sweet Banana on induction medium at day 7, 10, 14 and 16.

- (a) and (b) cells dividing in both periclinal and anticlinal directions in ground meristem (GM) of the cotyledon at day 7 (PD = protoderm)
- (c) globular structures developed on the cotyledons at day 10
- (d) globular structures developed on the cotyledons at day 14
- (e) and (f) globular structures developed on the cotyledons at day 16





Zimmerman (1993) and Dodeman et al. (1997). It seems that the entire protoderm of the globular somatic embryo structures gradually developed (emerged and grew embedded in the surface of the cotyledons) and finally attached to the cotyledons of the immature zygotic embryo explant. These structures were found developing at more than one specific location on the cotyledon of the same immature zygotic embryo explant and various sizes of the structures could be found on the same explant. Sometime root organogenesis could be found at the outer (abaxial) surface of the cotyledon (Plate 3.9 d).

On the germination or conversion medium, the globular somatic embryo structures developed more into what appeared to be heart, torpedo and cotyledonary stages (Plate 3.10) as previously described by Zimmerman (1993) and Dodeman et al. (1997). Sometime a secondary somatic embryo could be seen developing from another primary somatic embryo. However, plantlet germination had never been found on the hypocotyl, even though there were some what appeared to be somatic embryo structures.

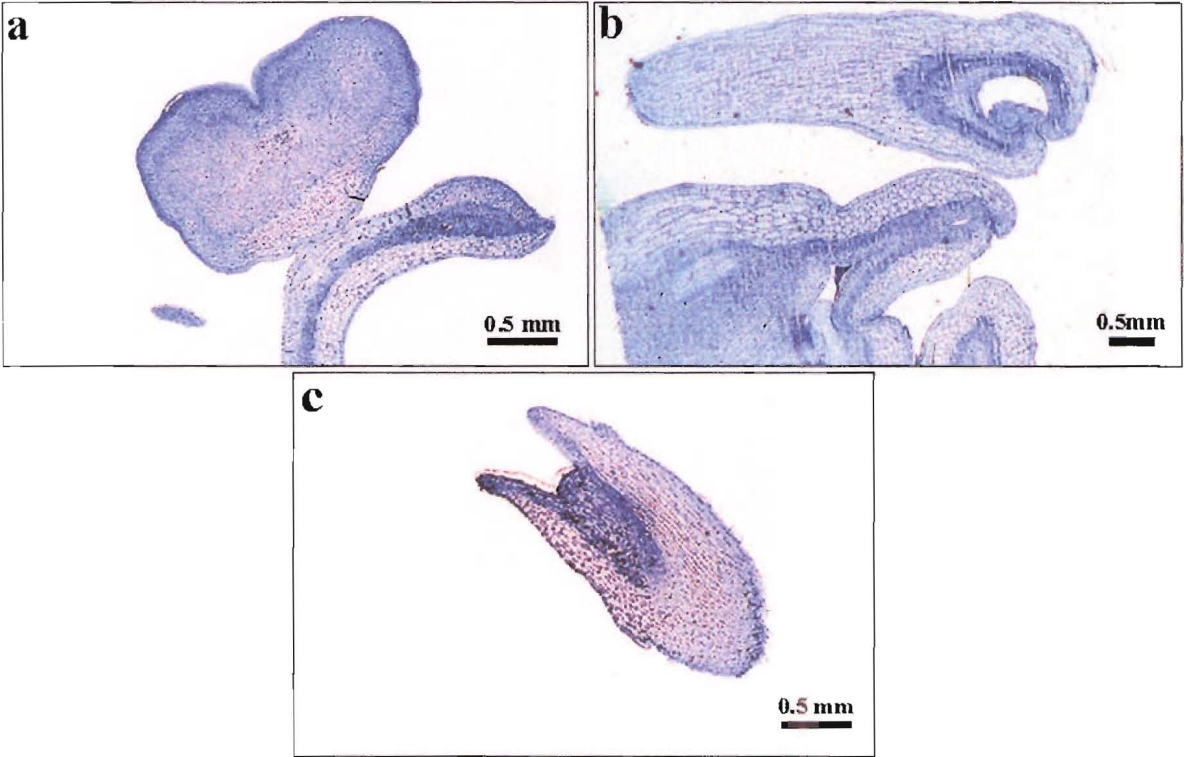
In summary, it has become clear from the present histological study that the primary event of interests occurring in the cotyledons of immature zygotic embryo explants on the somatic embryo induction medium was the formation of globular somatic embryos. After transfer to germination or conversion medium, more than somatic embryo germination or plantlet conversion was realised. On this medium, further development and maturation of the globular somatic embryos took place. The use of the name for this medium following widespread practice in the literature is clearly unsatisfactory and might have contributed to some unsubstantiated claims (without accompanying histological evidence) that somatic embryo initiation and maturation took place during culture on the somatic embryo induction medium (Harini and Lakshmi Sita, 1993; Binzel et al., 1996).

### **3.5 Growth of the plantlets obtained from germinated somatic embryos in soil (*C. annuum* L. cv. Sweet Banana)**

It was observed that many of the germinated somatic embryos formed callus and showed no further growth if they were left on the germination or conversion medium for more than 6-8 weeks. Upon further experiments using *C. annuum* L. cvs. Sweet Banana and Yolo Wonder another medium herein called plantlet development medium was found to be

**Plate 3.10** Histology of immature somatic embryo of *Capsicum annuum* L. cv. Sweet Banana on conversion medium.

- (a) a heart-shaped somatic embryo developed on the cotyledon at day 5
- (b) a torpedo-shaped somatic embryo developed on cotyledons at day 7
- (c) a cotyledonary-stage somatic embryo developed at day 10





advantageous in preventing callus formation and supporting plantlet growth and development following germination of somatic embryos.

To determine conditions for growing in soil, the plantlets derived from somatic embryogenesis were transferred from the plantlet development medium (Plate 3.11 a) to small pots (7 cm high X 7.5 cm diameter) containing potting mix (Plate 3.11 b). It was found that the plantlets required high humidity for successful acclimatisation since without being enclosed with a transparent plastic container, their leaves would wilt, turn yellow or drop and the whole plantlets collapsed later. With the plastic cover on for about 2 weeks, the plantlets developed more leaves and grew taller (Plate 3.11 b and c). Within 2 months after acclimatisation, the plants in the bigger pots (13 cm high X 12 cm diameter) began flowering and fruiting (Plate 3.11 d). The first fruit turned red 50 days later while more fruits were at different stages of development (Plate 3.11 e). The seeds obtained from these fruits appeared normal and could germinate to establish another fruiting crop. Overall, the plantlets of *C. annuum* L. cv. Sweet Banana regenerated from somatic embryogenesis did not show any major abnormalities in growth and development compared with the plants grown from natural seeds.

### **3.6 *In vitro* flowering of *C. annuum* L. cv. Sweet Banana**

Upon further culture on the plantlet development medium for three to four months, the plantlets formed floral buds and flowers (Plate 3.12). The *in vitro* flowers developed like those on plants in soil. However, these flowers mostly dropped after three to five days after anthesis.

*In vitro* flowering of many plant species has been achieved and applied to study the factors involved in the regulation of flowering (Scorza, 1982). Nonetheless, *in vitro* flowers might be smaller (Nadgauda et al., 1997) and malformed (Harada and Murai, 1998).

Here, for the first time, *in vitro* flowering of the plantlets regenerated via somatic embryogenesis in immature zygotic embryos of *C. annuum* L. cultivar Sweet Banana is reported. Furthermore, the *in vitro* flowers were examined more closely to see if they were

**Plate 3.11** Growth and development of somatic embryo-derived plants in soil.

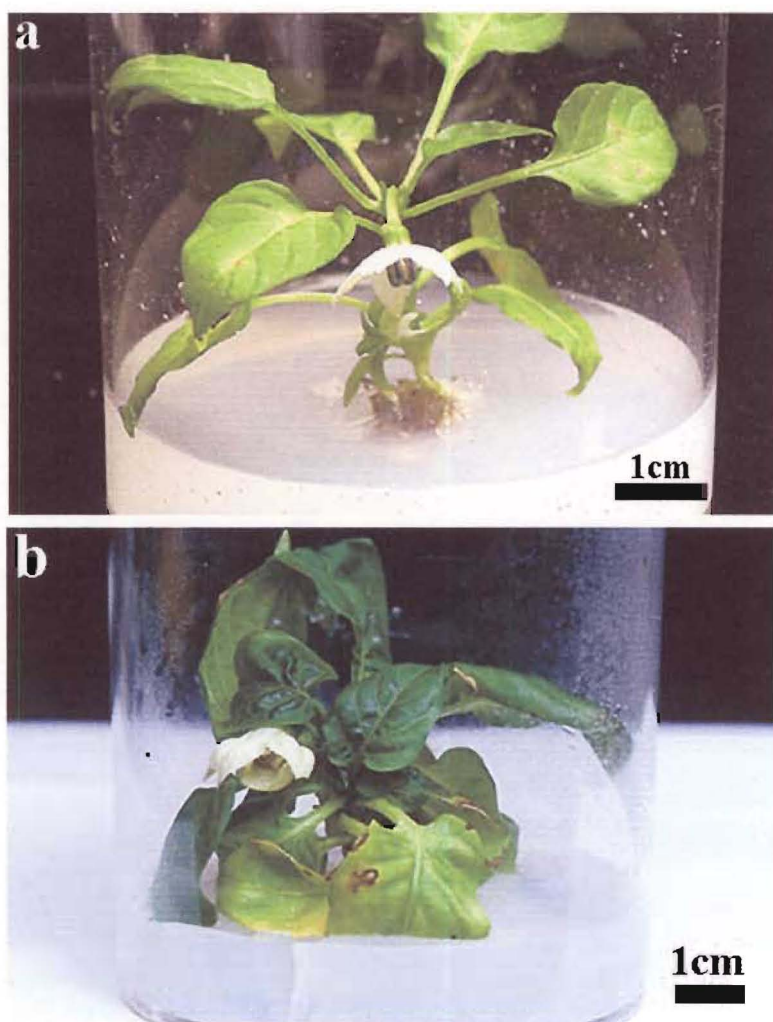
- (a) a plantlet of *Capsicum annuum* L. cv. Sweet Banana grown on plantlet development medium
- (b) 3 weeks after transfer to a small pot with potting mix
- (c) 6 weeks after ex-flasking
- (d) 9 weeks after ex-flasking
- (e) 15 weeks after ex-flasking



**Plate 3.12** *In vitro* flowering of plantlets regenerated from somatic embryogenesis of *Capsicum annuum* L.

(a) cv. Sweet Banana

(b) cv. Yolo Wonder



comparable to those produced by the mature plants grown in the glasshouse. This is also the first study comparing *in vitro* and *in vivo* flowers of *C. annuum* L.

### 3.6.1 Flower structures

*In vitro* and *in vivo* flowers of *C. annuum* L. cv. Sweet Banana were harvested for this comparative study on the day of their opening. The shape of both types of flowers appeared to be the same (Plate 3.13). A closer examination confirmed that there were no obvious differences in the outer parts (calyx colour, corolla number, corolla colour, and corolla length) of both *in vitro* and *in vivo* flowers (Table 3.8). However, a comparison of the inner parts of both types of flowers revealed that stamen number, anther length, filament length, carpel length and style length were slightly different (ANOVA,  $P < 0.05$ ) whereas anther colour, filament colour, carpel number, carpel colour, style colour, and stigma colour were the same (Table 3.8). The *in vitro* flowers tended to have more stamens, longer filaments and style, but smaller anthers and shorter ovary than *in vivo* flowers. This may be caused by *in vitro* condition or somaclonal variation of the flowers. The significance of these small differences in the flower parts to reproductive success is not known.

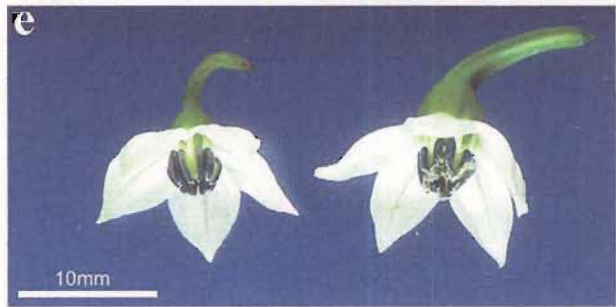
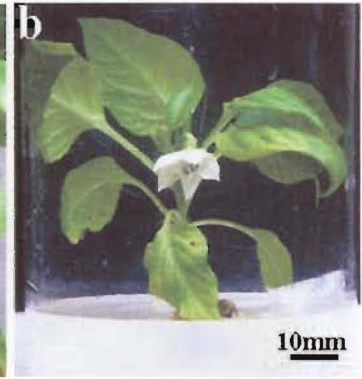
Overall, the data indicate that the *in vitro* flowers of *C. annuum* L. cv. Sweet Banana were well-formed and comparable to the *in vivo* flowers in many visual features. Similar findings were found in several recent studies e.g. *Leptinella nana* L. (Carson and Leung, 1994a), *Wahlenbergia stricta* L. (Carson and Leung, 1994b), bamboo (Nadgauda et al., 1997) and gentian (Zhang and Leung, 2000).

### 3.6.2 Pollen Morphology and Physiology

It has been suggested that pollen should be collected soon after anther dehiscence for morphological and physiological investigations (Shivanna and Rangaswamy, 1992). In general, the glasshouse-grown *C. annuum* L. cv. Sweet Banana plants released most of their pollen in the morning of flower opening. In contrast, the time of collecting pollen from the *in vitro* flowers was less predictable, presumably because the *in vitro* flowers were under 24 hours of continuous lighting in the growth room.

**Plate 3.13** Comparison of *in vitro* and *in vivo* flowers of *Capsicum annuum* L. cv. Sweet Banana. The *in vitro* flowers were produced by the plantlets regenerated via somatic embryogenesis.

- (a) development of tiny green floral buds from *in vitro* flowers
- (b) a well-formed, full-bloom mature *in vitro* flower. The pepper plantlet was grown in a culture container with its lid being left open temporarily daily over a period of 8 weeks
- (c) appearance of many tiny green floral buds of a pepper plant grown in a glasshouse
- (d) a well-formed, full-bloom mature *in vivo* flower
- (e) a close-up view of *in vitro* (on the left) and *in vivo* (on the right) flowers





**Table 3.8** Comparison of *in vitro* and *in vivo* flower characters of *Capsicum annuum* L. cv. Sweet Banana.

Outer parts\*

Source	Flower shape	Calyx colour	Corolla number	Corolla colour	Corolla length (mm)
<i>In vitro</i>	Bell type	Green	5.6±0.1a	White	9.4±0.3a
<i>In vivo</i>	Bell type	Green	5.3±0.1a	White	9.4±0.1a

Inner parts\*

Source	Stamen number	Anther colour	Anther length (mm)	Filament colour	Filament length (mm)	Carpel number	Carpel colour	Carpel length (mm)	Style colour	Style length (mm)	Stigma colour
<i>In vitro</i>	5.8±0.2a	Purple	2.6±0.1a	White	2.22±0.1a	1	Green	6.3±0.3a	White	3.4±0.2a	Green
<i>In vivo</i>	5.3±0.1b	Purple	3.1±0.03b	White	2.21± 0.1b	1	Green	6.8±0.1b	White	2.8±0.1b	Green

\*Values are means of 20 replicates ± S.E. from *in vitro* flowers and 50 replicates ± SE from *in vivo* flowers on the day of their opening. Data marked by same letter in a column are not significantly different (ANOVA,  $P < 0.05$ ).

There are few publications on pollen of the Solanaceae (Punt and Monna-Brands, 1977), especially pollen morphology of *C. annuum* L. The present SEM observations revealed that the morphology of pollen from both *in vitro* and *in vivo* flowers is not different (Table 3.9). Both *in vitro* and *in vivo* pollen grains are not united in-group. Grains have three colpi (elongate apertures) and pori (circular apertures) arranged in an equatorial zone. This is similar to the descriptions given in the Moore's pollen and spore key (Moore et al., 1991). Shape of symmetrical grain in polar view is circular but elliptic in equatorial view (Plate 3.14).

Using Alexander's staining method (1969), it was found that over 90% of the pollen from both sources of flowers were viable (Plate 3.15 a-d). However, the pollen grains from *in vitro* flowers were marginally less viable than those from *in vivo* flowers (Table 3.9).

The *in vitro* germination test showed that the pollen of *in vivo* flowers performed better than those of *in vitro* flowers (Table 3.9). Brewbaker and Kwack (1963) reported that the percentage of germination in *Capsicum* varied from 2-35%. In this study with the cultivar Sweet Banana, about 34 and 25% of the pollen from *in vivo* and *in vitro* flowers germinated (Table 3.9), respectively, although Mercado et al. (1994) used the same medium and observed *Capsicum* pollen germination close to 50%. Pollen of *in vitro* flowers germinated slower than those of *in vivo* flowers. No germination of pollen from either source resulted after 15 hours from the start of the germination test (Plate 3.15 e and f). This was different from another study showing that no pollen of *C. annuum* L. F<sub>1</sub> hybrid 'Latino' germinated after 6 hours. This suggested that pollen germination may vary among pepper cultivars.

This is the first time that not only *in vitro* flowering of a cultivar of *C. annuum* L. can be induced in plantlets regenerated via somatic embryogenesis but also the parts of the *in vitro* flowers largely appear normal and their pollen are highly viable and germinable.

**Table 3.9** Comparison of the morphology, viability and germination of pollen grains from *in vitro* and *in vivo* flowers of *Capsicum annuum* L. cv. Sweet Banana.\*

Source	Pollen morphology (from SEM)					Pollen physiology	
	Pollen type	Grain shape		Exine sculpture	Pollen length in equatorial view ( $\mu\text{M}$ )	Pollen viability (%)	Pollen germination** (%)
		Equatorial view	Polar view				
<i>In vitro</i>	Trizonocolporate	Elliptic	Circular	Scabrate	39.4 $\pm$ 0.2a	94.2 $\pm$ 0.9a	25.2 $\pm$ 1.4a
<i>In vivo</i>	Trizonocolporate	Elliptic	Circular	Scabrate	39.9 $\pm$ 0.2a	96.3 $\pm$ 0.5b	34.4 $\pm$ 1.8b

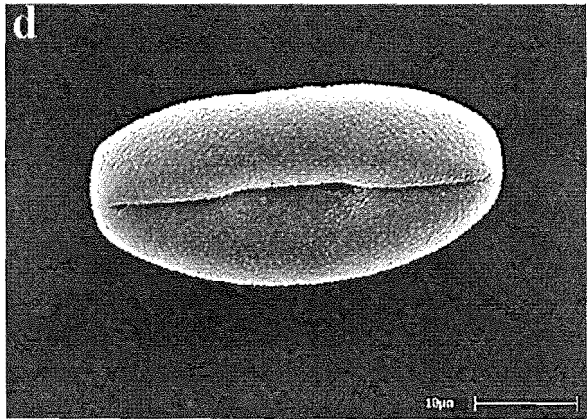
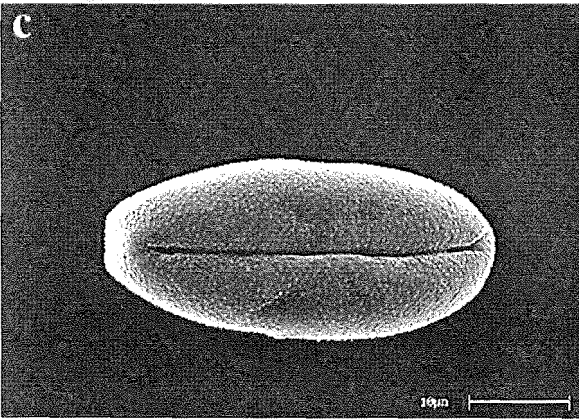
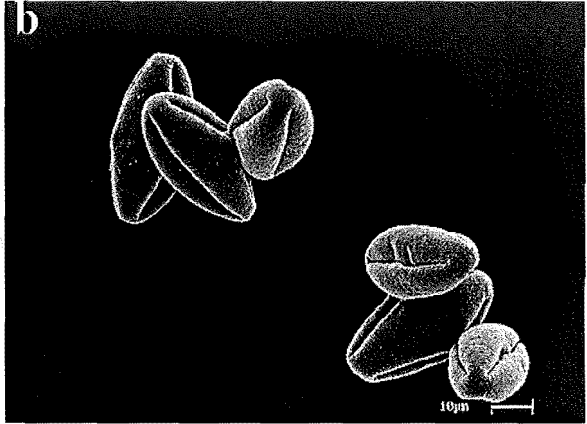
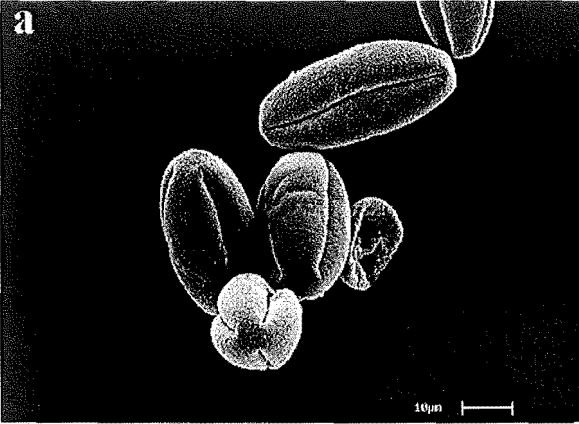
\*Values are means of 60 pollen grains  $\pm$  S.E. from *in vitro* and *in vivo* flowers on the day of their opening except those of pollen viability and pollen germination in which pollen grains from 6 replicate flowers were used. Data marked by same letter in a column are not significantly different (ANOVA,  $P < 0.05$ ).

\*\*Pollen from *in vitro* and *in vivo* flowers began to germinate 45 and 30 minutes, respectively, after the start of the germination test. Data were obtained after 24 hours later.

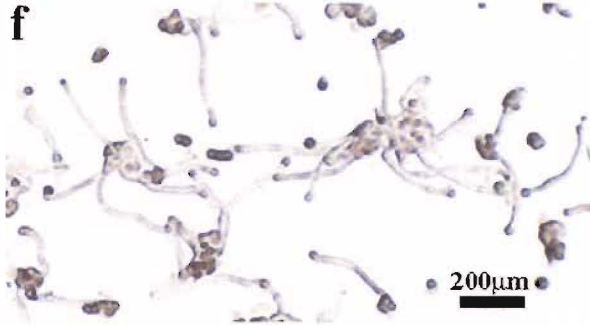
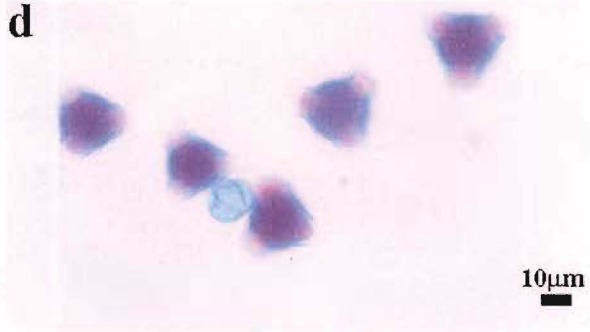
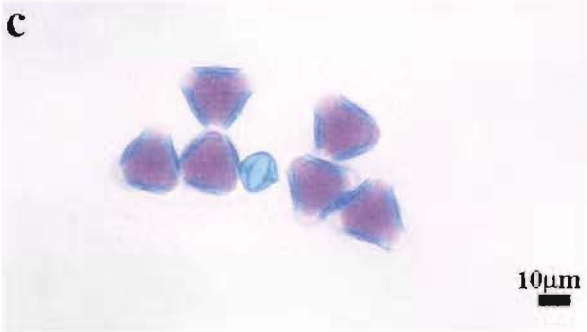
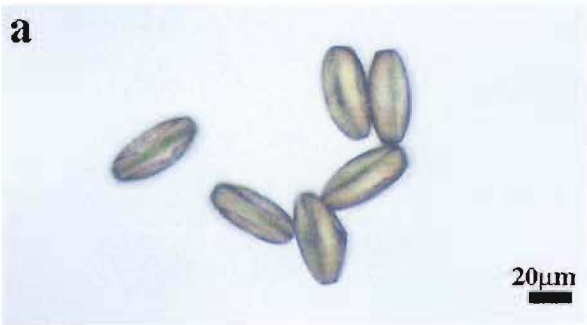
**Plate 3.14** SEM observations of pollen grains from *in vitro* and *in vivo* flowers of *Capsicum annuum* L. cv. Sweet Banana.

(a) and (c) pollen of *in vitro* flowers

(b) and (d) pollen of *in vivo* flowers



**Plate 3.15** Viability and germinability tests on pollen from *in vitro* and *in vivo* flowers of *Capsicum annuum* L. cv. Sweet Banana. Pollen from *in vitro* flowers before (a) and after (c) staining for viability test; pollen from *in vivo* flowers before (b) and after (d) staining for viability test. Germination of pollen from *in vitro* (e) and *in vivo* (f) flowers after 15 hours from sowing on Mercado's medium (see Appendix 7).



### 3.7 *In vitro* fruiting of *C. annuum* L. cv. Sweet Banana

*In vitro* flowering of many plant species has been reported, for example, *Leptinella nana* L. (Carson and Leung, 1994a), *Wahlenbergia stricta* L. (Carson and Leung, 1994b), bamboo (Nadgauda et al., 1997), chicory (Demeulemeester and De Proft, 1999), *Murraya paniculata* (L.) Jack (Jumin and Ahmad, 1999) and gentian (Zhang and Leung, 2000) and many more in the older literature (Scorza, 1982). By comparison, reports on *in vitro* fruiting are relatively scarce e.g. groundnut (Narasimhulu and Reddy, 1984), cauliflower (Kumar et al., 1995) *Capsicum frutescens* L. (Tisserat and Galletta, 1995), strawberry (Asao et al., 1997), *Vigna mungo* (L.) Hepper (Ignacimuthu et al., 1997) and pea (Fujioka et al., 1999; Franklin et al., 2000b).

Culturing shoot tips derived from seedlings of *Capsicum frutescens* L. in a relatively large aseptic container (capacity of 6 litres) with liquid medium resulted in the formation of *in vitro* flowers, few of which subsequently developed into fruits (Tisserat and Galletta, 1995). However, it is not clear if the *in vitro* pepper fruits contained viable seeds. It is also not known if hand-pollination is beneficial for seed setting under *in vitro* conditions.

During this research on somatic embryogenesis in *C. annuum* L. cv. Sweet Banana, *in vitro* flowering of the regenerated plantlets was observed. Here, using this *in vitro* flowering system, the experiments overcoming the problems associated with the growth and development of flowers, fruits and seeds all under *in vitro* conditions in small tissue culture containers (capacity of 750 ml) are described.

#### 3.7.1 From immature floral buds to fruitlet formation

During the ongoing investigations into somatic embryogenesis starting from immature zygotic embryos of *C. annuum* L. cv. Sweet Banana, the regenerated plantlets were found to form tiny green floral buds (Plate 3.13 a) after about 2 months of culture in tissue culture containers (8.5 cm high X 6.5 cm diameter or 15 cm high X 7 cm diameter) with MS basal medium supplemented with 1 mg/l NAA.



Within 5-7 days of their formation, most of these tiny floral buds of the plantlets kept in the closed tissue culture containers either turned brown or dropped during their development. Only occasionally did few of them grow further to white floral buds or even to the mature stage of flower anthesis (Table 3.10).

Contrary to the notion that exogenous auxin could act as a principal floral inhibitor (Scorza, 1982), the result here shows that NAA in the medium did not prevent *in vitro* flower formation. This was also found in other studies (for example, Ignacimuthu et al., 1997; Franklin et al., 2000).

Accumulation of volatile substances such as ethylene in the small, closed tissue culture containers used in the present research might hinder further growth and development of the immature floral buds formed *in vitro*. The following results are consistent with this suggestion. The lids of at least 5 tissue culture containers (15 cm high X 7 cm diameter) each with a pepper plantlet bearing a green tiny floral bud were left open 15-20 minutes inside a laminar flow cabinet everyday or every two days over a period of 2 weeks. It was found that most of the tiny floral buds (at least 5) could develop into well-formed, full-bloom mature flowers (Plate 3.13 b, Table 3.10). However, these flowers could only grow to become tiny green fruits which abscised soon after they were formed.

The vented autoclavable plant tissue culture containers (Phytakon™) were trialled to see if passive but continuous changes in gaseous environment surrounding the *Capsicum* plantlets would permit the further development of the *in vitro* flowers. It was found that not only the number of mature flowers formed per plant was significantly higher than the other 2 treatments (Plate 3.16 a, Table 3.10), the flowers (at least 30 studied) could also grow further and develop into tiny green fruits (Plate 3.16 b) which still abscised prematurely in a few days later. Improved air flow has also been found to be beneficial in other *in vitro* flowering systems (Tisserat and Galletta, 1995; Asao et al., 1997; Fujioka et al., 1999).

### 3.7.2 *In vitro* ripening of pepper fruits

A well-known action of ethylene is stimulation of flower senescence (Sisler and Yang, 1987). In *C. annuum* plants grown in the glasshouse, it was found that application of silver thiosulfate ( $\text{Ag}_2\text{S}_2\text{O}_3$ ), an inhibitor of ethylene action, suppressed flower abscission (Aloni

**Table 3.10** Importance of ventilation to *in vitro* flowering of pepper plantlets (*Capsicum annuum* L. cv. Sweet Banana).

Treatment	% of flowering <sup>1</sup>	No. flowers/plantlet <sup>2</sup>
I. Closed culture system	4.2 <sup>a</sup>	0.04± 0.02 <sup>a</sup>
II. Temporary opening of culture container	100 <sup>b</sup>	1 <sup>b</sup>
III. Phytakon <sup>TM</sup> culture container	100 <sup>b</sup>	3.9± 0.4 <sup>c</sup>

Data were obtained from 3 replicate experiments and the means ± S.E. were calculated. Within a column the results marked by the same letter are not significantly different [ANOVA,  $P < 0.05$ ; Tukey (HSD) comparison of means].

1: % of plantlets forming at least 1 mature flower which may or may not develop further

2: only mature fully open flowers (same as those in Plate 3.16 a) formed within 8 weeks were counted

Treatment I: tissue culture containers (8.5 cm high X 6.5 cm diameter or 15 cm high X 7 cm diameter) with their lids kept closed for the entire duration of the experiment (8 weeks); n = 120.

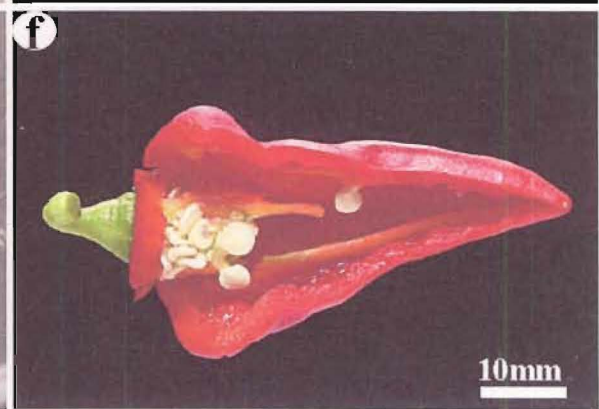
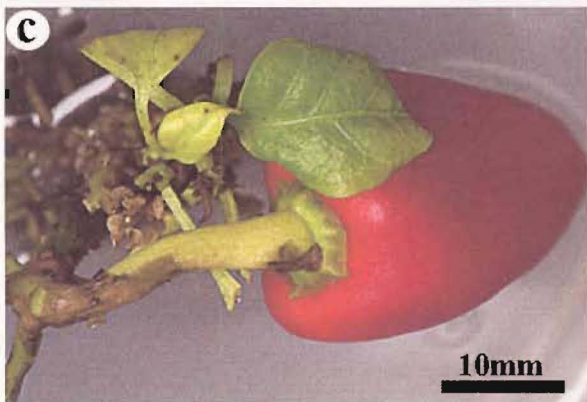
Treatment II: the lid of tissue culture containers (15 cm high X 7 cm diameter) was left open daily for 15 minutes under aseptic conditions in a laminar flow cabinet; n = 5.

Treatment III: n = 16.

In all 3 treatments, each container had 1 plantlet grown on the plantlet development medium.

**Plate 3.16** From *in vitro* flowers to *in vitro* fruits of *Capsicum annuum* L. cv. Sweet Banana.

- (a) *in vitro* well-formed, full-bloom mature flowers of a pepper plantlet grown in a Phytakon<sup>TM</sup> culture container
- (b) a tiny green fruit of a pepper plantlet grown in a Phytakon<sup>TM</sup> culture container
- (c) an abnormal *in vitro* red fruit from the  $\text{Ag}_2\text{S}_2\text{O}_3$  treatment
- (d) abnormal-looking seeds formed inside an abnormal *in vitro* red fruit
- (e) *in vitro* long tapered red fruit developed after hand-pollination treatment
- (f) normal-looking seeds formed inside a long tapered red fruit formed *in vitro*



et al., 1995) and reduced fruit abscission (Hoyer, 1998). It was hypothesised here that ethylene level in the Phytakon<sup>TM</sup> containers might still be too inhibitory for further growth and development of the young pepper fruits. To test this possibility, inhibitors of ethylene actions such as silver nitrate ( $\text{AgNO}_3$ ) and silver thiosulfate ( $\text{Ag}_2\text{S}_2\text{O}_3$ ) (Zobayed et al., 1999) were added to the plantlet development medium in the following ways. Initially, liquid MS basal medium containing 20  $\mu\text{M}$   $\text{AgNO}_3$  with or without 3% sucrose was added directly to the surface of the agar-gelled plantlet development medium in the Phytakon<sup>TM</sup> containers. It was found that all the tiny green fruits (at least 20 studied) still dropped after a few days of fruit setting (Table 3.11). It is possible that  $\text{AgNO}_3$  was not translocated to the developing fruits as it is not known to be too mobile within the plant (Perl et al., 1988). Subsequently,  $\text{Ag}_2\text{S}_2\text{O}_3$  which is a more translocatable form of ethylene action inhibitor (Veen, 1983; Aloni et al., 1995; Hoyer, 1998) was trialled. After adding this solution, more than 20 *in vitro* flowers studied were well-formed and comparable to the *in vivo* flowers in many visual features. Furthermore, close to 20% of the resulting green fruits increased in size until 55 days when they turned red (Plate 3.16 c, Table 3.11). Apart from turning completely red, which is an important indicator of fruit ripening, the *in vitro* fruits were quite small (Table 3.11) and of conical shape, unlike the long tapered fruits formed on the glasshouse-grown plants. Also unlike the normal fruits from the glasshouse-grown plants, the *in vitro* fruits contained no or few abnormal looking seeds which did not germinate (Plate 3.16 d).

### 3.7.3 Effect of hand-pollination

Although *C. annuum* L. is classified mainly as a self-pollinating plant (Tanksley, 1984), the *in vitro* condition may lead to poor self-pollination. In this experiment, hand-pollination was undertaken to see if there is any beneficial effect on seed setting. After *in vitro* flower anthesis, the anthers were harvested aseptically in a laminar flow cabinet and were kept in a sterile Petri dish to collect pollen which was stored at 4 °C for 1 month. Preliminary experiment had shown that the pollen grains could still germinate after a month of storage. When other *in vitro* flowers were fully open, the pollen previously collected and stored at 4 °C was used for hand-pollination under aseptic conditions in a laminar flow cabinet. As a result, the *in vitro* fruits (at least 6 studied) had long tapering shape (Plate 3.16 e) similar to those grown in the glasshouse (Table 3.11). The largest fruit

**Table 3.11** Effect of silver nitrate ( $\text{AgNO}_3$ ), silver thiosulfate ( $\text{Ag}_2\text{S}_2\text{O}_3$ ) and hand-pollination on *in vitro* fruiting of *Capsicum annuum* L. cv. Sweet Banana.

Treatment	% of plantlets forming a red fruit	No. red fruit/plantlet	Days of fruit ripening <sup>1</sup>	fruit length <sup>2</sup> (cm)
$\text{AgNO}_3$ n = 16	0	0	0	0
$\text{Ag}_2\text{S}_2\text{O}_3$ n = 16	18.6 <sup>a</sup>	0.2 ± 0.1 <sup>a</sup>	46 ± 4.6 <sup>a</sup>	2.2 ± 0.2 <sup>a</sup>
Hand-pollination n = 6	100 <sup>b</sup>	1 <sup>b</sup>	48 ± 2.7 <sup>a</sup>	5.3 ± 0.5 <sup>b</sup>

Data were obtained from 3 repeat experiments and the means ± S.E. were calculated. Those within a column marked by the same letter are not significantly different [ANOVA,  $P < 0.05$ ; Tukey (HSD) comparison of means].

1: time for fruits to turn completely red.

2: measured at the end of experiment; duration of experiment = 60 days

formed was 7.1 cm long. Usually each plantlet bore 1 red, presumably ripe fruit. After fruit setting some flowers were still formed (at least 10 studied) but they could only develop to the tiny green fruit stage. These tiny fruits still dropped after a few days of fruit setting.

The *in vitro* fruits began to turn red around 50 days and in less than 6 days later were fully red. Compared with the fruits formed *in vitro* without hand-pollination, those formed following *in vitro* hand-pollination appeared to have a far greater number of seeds and many of them seemed to be of similar appearance and size to those found inside the red fruits from glasshouse-grown plants. The *in vitro* fruits were smaller presumably because they matured earlier than the glasshouse-grown ones. For example, an *in vitro* fruit cut open after 65 days was found to contain 16 normal-looking seeds (Plate 3.16 f) and 18 tiny, abnormal-looking ones. When 3 of the normal-looking seeds were tested for germination in a dark room at 26 °C for 7 days, none of them germinated although the embryos inside were viable as they stained red with 0.5% (w/v) tetrazolium chloride. These seeds appeared to lack a hard endosperm, suggesting that they were probably immature and had not attained the capacity for germination. One of the *in vitro* fruit was definitely developed from an *in vitro* flower with its pre-dehiscent anthers removed before hand-assisted cross pollination with pollen from another flower.

The result of Tisserat and Galletta (1995) was different from the present experiment in that they found no flowering from the plantlets of *Capsicum frutescens* L. grown on agar-based medium. Also while *in vitro* pod and seed formation of cauliflower (Kumar et al., 1995) and *in vitro* fruiting and formation of viable seed in green pea (Franklin et al., 2000) could occur in the absence of hand-pollination, it is found here that hand-pollination was beneficial to *in vitro* fruit development and seed setting of pepper plantlets. Similarly, *in vitro* strawberry fruits were obtained only following hand-pollination (Asao et al., 1997).

Moreover, *in vitro* fruiting of *Capsicum frutescens* L. was formed only in the large culture chamber with the air ventilation system (Tisserat and Galletta, 1995). This outcome was quite similar to *in vitro* fruiting of strawberry (Asao et al., 1997) and pod setting of pea (Fujioka et al., 1999).

Fujioka et al. (1999) achieved 100% of *in vitro* pea flowering when large test tubes (30X200 mm) were used instead of the smaller containers (25X150 mm). Similarly, *in*

*vitro* flowering of strawberry was achieved when the culture container was changed (Asao et al., 1997) from a flask (300 ml) to a culture-bottle (100mmX110mmX100mm) with 2 pieces of air (flowable) filter (8mm diameter and 0.02µm pore size) on the lid for better airflow condition.

In conclusion, this is the first report showing that in small tissue culture containers pepper plantlets regenerated from immature zygotic embryos could form *in vitro* flowers, some of which developed into ripe fruits with viable seeds. Some of the critical factors seem to include improved air flow, inhibition of ethylene-induced fruit abscission and the beneficial effect of hand pollination. Further research is required to determine why the *in vitro* seeds, though viable, failed to germinate (see later 3.8.4 section). The present fruiting system might aid studies into the molecular physiology of fruit ripening in pepper.

### **3.8 Clonal propagation of the plantlets of *C. annuum* L. cv. Sweet Banana which were regenerated via somatic embryogenesis**

#### **3.8.1 Preliminary study on clonal propagation**

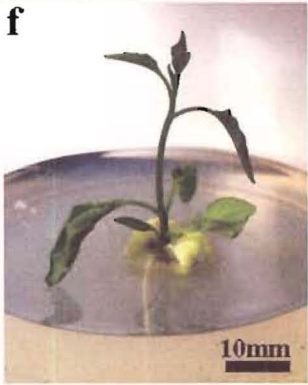
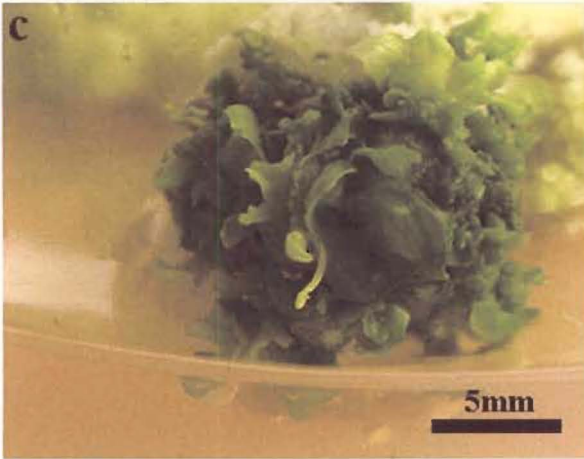
The pepper clonal propagation protocol of Husain et al. (1999) was initially trialled using nodal segments (6-8 mm long) excised from the plantlets of *C. annuum* L. cv. Sweet Banana which were regenerated via somatic embryogenesis and maintained on the plantlet development medium. Within one month of culture on MS basal medium supplemented with 5 mg/l BA, 2 mg/l PAA and 3% (w/v) sucrose, development of new shoots occurred in most segments (Plate 3.17 a). However, shoot elongation did not occur even when the nodal explants were transferred once to the same medium or one of the same composition except with lower levels of BA (2 mg/l) and PAA (1 mg/l) (Plate 3.17 b). Moreover, some nodal explants developed green compact calli and clusters of leafy-like structures (Plate 3.17 c and d). The present result is different from that of Husain et al. (1999) who used cotyledon explants.

A different medium aiming to improve shoot elongation was trialled basing on the general physiological effect of GA<sub>3</sub> on shoot growth. It was found that medium containing 5 mg/l GA<sub>3</sub> obviously increased shoot elongation (Plate 3.17 e) and was better than that containing 10 mg/l GA<sub>3</sub> medium. After elongation some shoots could form roots on the



**Plate 3.17** Preliminary study on clonal propagation using nodal explants (6-8 mm long) of *Capsicum annuum* L. cv. Sweet Banana. The explants were excised from the plantlets which were regenerated via somatic embryogenesis and maintained on the plantlet development medium.

- (a) after one month of culture on MS basal medium supplemented with 5 mg/l BA, 2 mg/l PAA and 3% (w/v) sucrose
- (b) the culture from (a) was transferred to a new medium with reduced levels of BA and PAA, i.e. 2 and 1 mg/l, respectively
- (c) development of leaf-like structures and callus after 1 month of culture on MS basal medium supplemented with 5 mg/l BA, 2 mg/l PAA and 3% (w/v) sucrose
- (d) development of leaf-like structures after the culture from (c) was transferred to the same medium as in (b)
- (e) elongation of shoots on MS basal medium supplemented with 5 mg/l GA<sub>3</sub> plus 3% (w/v) sucrose
- (f) rooting on MS basal medium supplemented with 5 mg/l GA<sub>3</sub> plus 3% (w/v) sucrose



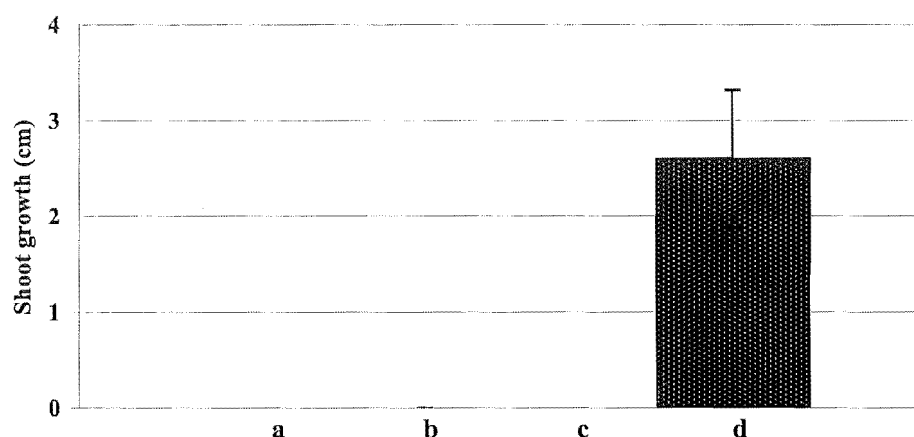
medium containing 5 mg/l GA<sub>3</sub> (Plate 3.17 f), suggesting that endogenous auxin was present at a sufficient level to initiate adventitious root formation. Details of additional preliminary experiments are found in Appendix 8. The following experiments were designed to show that exogenous growth regulators are not required for clonal propagation of *Capsicum* plantlets. Instead, sucrose in the medium appears to be an important factor.

### **3.8.2 Clonal propagation using nodal segments and shoot tips of *C. annuum* L. cv. Sweet Banana plantlets which were regenerated via somatic embryogenesis**

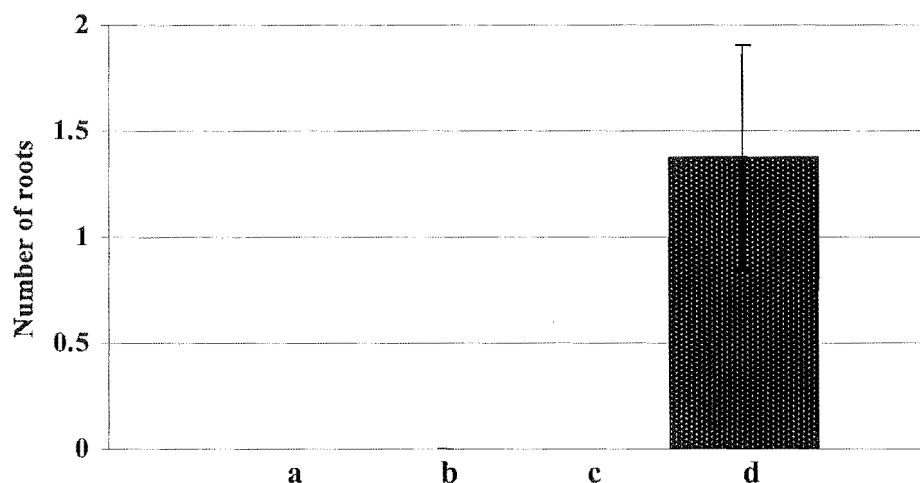
Shoot elongation and root formation did not occur when nodal and excised shoot tip explants were cultured on sucrose-free MS basal medium for 6 weeks (Figure 3.1, 3.2 and Plate 3.18 a). Instead a white-watery callus was formed on one side of the nodal explants. When cultured on sucrose-free medium, only shoot tip explants exhibited some leaf development (Figure 3.3) and callus formation was not found. However, the number of leaves was greatly increased on both nodal and shoot tip explants after they were transferred to medium supplemented with 3% (w/v) sucrose (Figure 3.3 and Plate 3.18 b).

Transfer of nodal explants from sucrose-free medium to one containing 3% sucrose did not result in shoot development nor root formation. In contrast, a completely different result was obtained from the same transfer experiment using shoot tip explants (Figure 3.1 and 3.2). It seems that for shoot, root and leaf development the supply of sucrose in the medium may be particularly important during initial stages of nodal explant culture. The result of placing nodal or shoot tip explants from the start of culture on medium containing either 3 or 5% sucrose are consistent with this suggestion (Figure 3.4 A-D and Plate 3.18 c-f). In general, shoot tip explants appeared to develop better than nodal explants on the sucrose-containing media. It is also worth noting that root and leaf development in shoot tip explants seemed to be better in 5% than 3% sucrose medium, particularly after 14 weeks of culture.

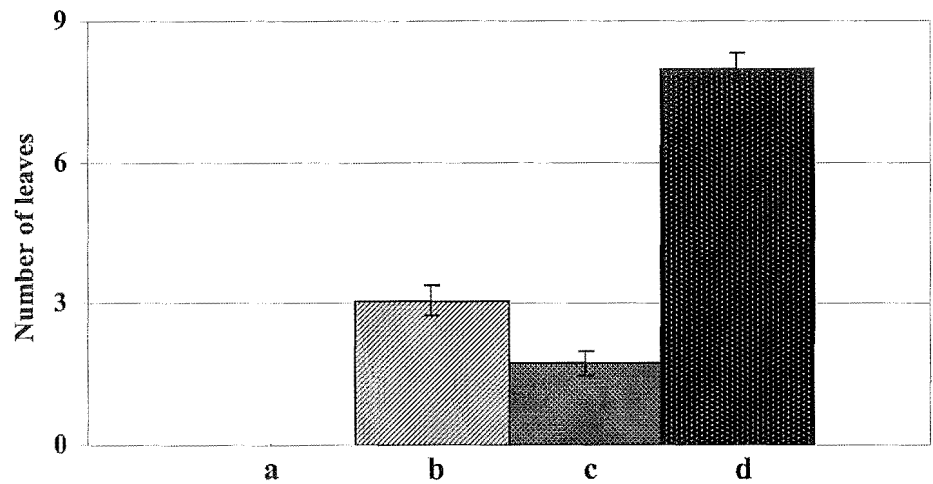
In summary, externally supplied plant growth regulator was not required for shoot and root development from nodal and excised shoot tip explants of *C. annuum* L. cv. Sweet Banana. Instead, sucrose was found to be important. There is no need to design separate media for shoot and root development in both types of explants. This is in



**Figure 3.1** Requirement of sucrose for shoot growth in nodal (a and b) and shoot tip (c and d) explants from somatic embryo-derived plantlets of *Capsicum annuum* L. cv. Sweet Banana. The explants were cultured on plant growth regulator-free MS basal medium without sucrose for 6 weeks (a and c) before they were transferred to the same growth regulator-free medium but supplemented with 3% sucrose (b and d). After transfer, the explants were cultured for another 8 weeks before the mean shoot lengths  $\pm$  SE were determined. This experiment was repeated 3 times. In a replicate experiment, 15 nodal and 8 shoot tip explants were used. The results in this figure were obtained from a replicate experiment.



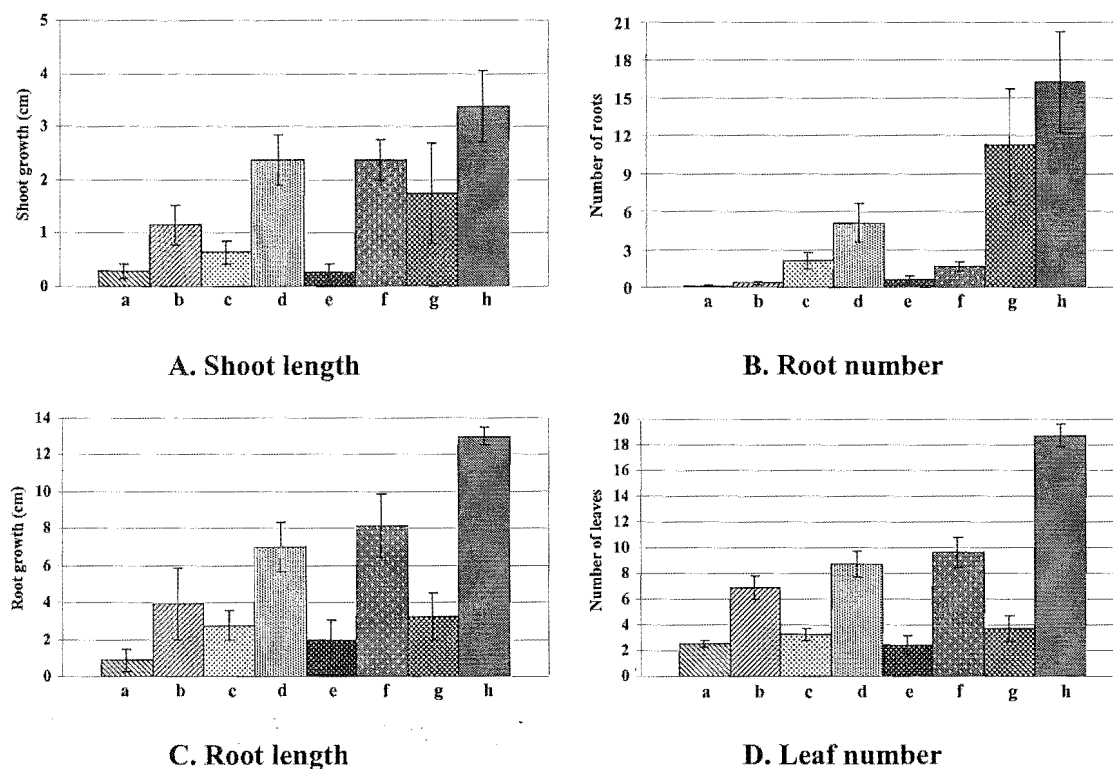
**Figure 3.2** Requirement of sucrose for root formation in nodal (a and b) and shoot tip (c and d) explants from somatic embryo-derived plantlets of *Capsicum annuum* L. cv. Sweet Banana. The explants were cultured on plant growth regulator-free MS basal medium without sucrose for 6 weeks (a and c) before they were transferred to the same growth regulator-free medium but supplemented with 3% sucrose (b and d). After transfer, the explants were cultured for another 8 weeks before the results (mean number of roots  $\pm$  SE) were obtained. This experiment was repeated 3 times. In a replicate experiment, 15 nodal and 8 shoot tip explants were used. The results in this figure were obtained from a replicate experiment.



**Figure 3.3** Effect of sucrose on number of leaves formed in nodal (a and b) and shoot tip (c and d) explants from somatic embryo-derived plantlets of *Capsicum annuum* L. cv. Sweet Banana. The explants were cultured on plant growth regulator-free MS basal medium without sucrose for 6 weeks (a and c) before they were transferred to the same growth regulator-free medium but supplemented with 3% sucrose (b and d). After transfer, the explants were cultured for another 8 weeks before the results (mean number of leaves  $\pm$  SE) were obtained. This experiment was repeated 3 times. In a replicate experiment, 15 nodal and 8 shoot tip explants were used. The results in this figure were obtained from a replicate experiment.

Statistical analysis [ANOVA,  $P < 0.05$ ; Tukey (HSD) test]

<b>Figure 3.3:</b>	a is significantly different from b, c and d
	b is significantly different from c and d
	c is significantly different from d



**Figure 3.4 A-D** Development of nodal (a-d) and shoot tip (e-h) explants from somatic embryo-derived plantlets of *Capsicum annuum* L. cv. Sweet Banana. The explants were cultured on MS basal medium containing 3% sucrose for 6 weeks (a and e) and 14 weeks (b and f), or 5% sucrose for 6 weeks (c and g) and 14 weeks (d and h). This experiment was repeated 3 times. In a replicate experiment, 15 nodal and 8 shoot tip explants were used. Means  $\pm$  SE of data from a replicate experiment are presented.

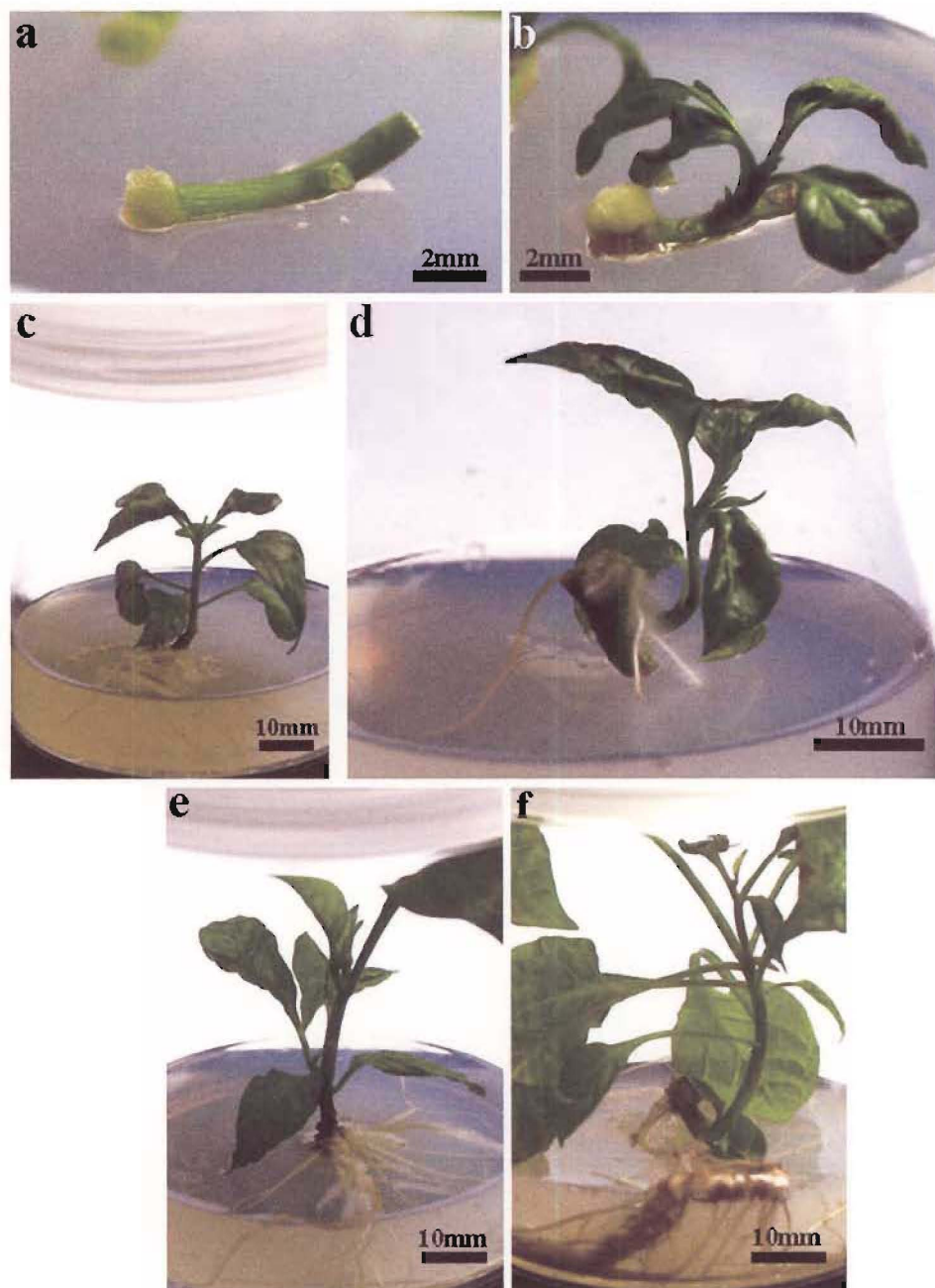
Statistical analysis [ANOVA,  $P < 0.05$ ; Tukey (HSD) test]

<b>A:</b>	a-c and e are significantly different from d, f, g and h
<b>B:</b>	a-f are significantly different from h a-c and e-f are significantly different from g
<b>C:</b>	a-g are significantly different from h a-c, e and g are significantly different from f a and e are significantly different from d
<b>D:</b>	a-g are significantly different from h a, c, e and g are significantly different from d and f b is significantly different from e

**Plate 3.18** Response of nodal and excised shoot tip explants from the somatic embryo-derived plantlets of *Capsicum annuum* L. cv. Sweet Banana on plant growth regulator-free medium supplemented with 3% or 5% (w/v) sucrose.

- (a) a nodal explant cultured on medium without sucrose for 6 weeks
- (b) transfer of (a) to medium supplemented with 3% (w/v) sucrose for further culture of 8 weeks
- (c) a shoot tip explant on medium supplemented with 3% (w/v) sucrose for 6 weeks
- (d) a nodal explant on medium supplemented with 3% (w/v) sucrose for 6 weeks
- (e) a shoot tip explant on medium supplemented with 5% (w/v) sucrose for 6 weeks
- (f) a nodal explant on medium supplemented with 5% (w/v) sucrose for 9 weeks





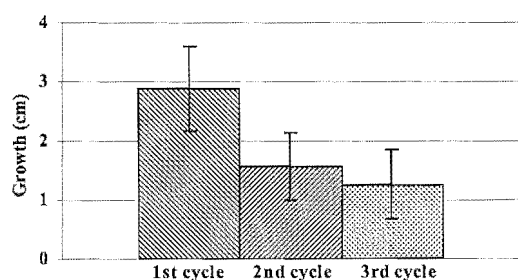
agreement with a previous and the only report using plant growth regulator-free medium for *Capsicum frutescens* L. shoot-tip explant propagation (Tisserat and Galletta, 1995).

### **3.8.3 Prospect of multiple cycles of clonal propagation using nodal and shoot tip explants from *C. annuum* L. cv. Sweet Banana plantlets**

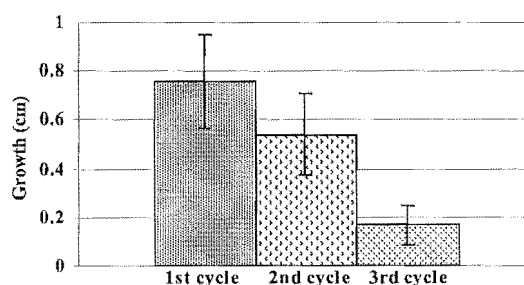
Having established the beneficial effect of sucrose for shoot and root development (i.e. whole plantlet regeneration) starting with nodal and shoot tip explants, it was of great interest to determine the potential for repeating this cycle of *in vitro* propagation (i.e. clonal propagation) many times. With this in mind, an experiment was carried out starting with nodal and shoot tip explants from somatic embryo-derived plantlets for the first cycle of plantlet regeneration. The shoots of the resulting plantlets in this cycle were divided into nodal and shoot tip explants for the next cycle of plantlet regeneration. At least 2 successive cycles of propagation were completed. The result showed that the performance of the explants was largely similar in successive cycle of propagation (Figure 3.5 and 3.6). The present results are promising in that multiple cycles of clonal propagation of plantlets regenerated via somatic embryogenesis in *Capsicum* seem to be possible. In the future, many more cycles of propagation are needed to confirm the reliability of the clonal propagation protocol described here. Furthermore, although sucrose has been found to be an important factor here, the requirement for an externally supplied carbohydrate needs to be examined more closely and in greater details. The importance of other factors including light intensity and its interaction with externally supplied carbohydrate, has yet to be determined.

### **3.8.4 *In vitro* flowering and fruiting of plantlets of *C. annuum* L. cv. Sweet Banana during clonal propagation**

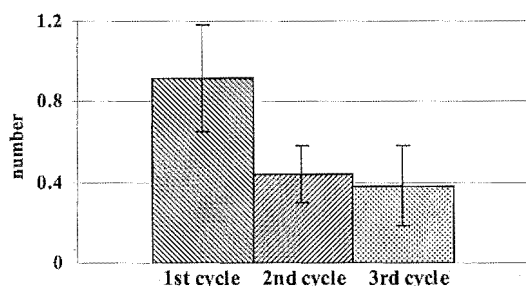
An interesting observation during the development of clonal propagation protocol was that if the plantlets regenerated from nodal and shoot tip explants were left on the same medium containing 3 or 5% sucrose for 14 weeks, they were capable of *in vitro* flowering and fruiting. They formed flowers and tiny fruits even they were kept in a small container (8.5 cm high X 6.5 cm diameter) and without hand pollination. However, these *in vitro* flowers and fruits mostly abscised a few days after their formation. To attempt to



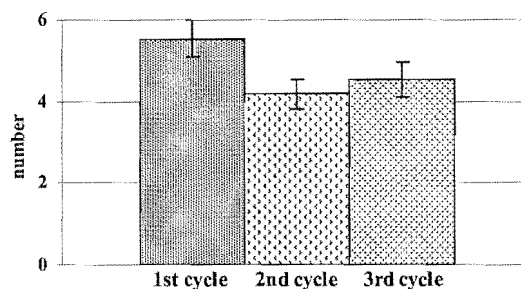
A. Root growth



B. Shoot growth



C. Root number

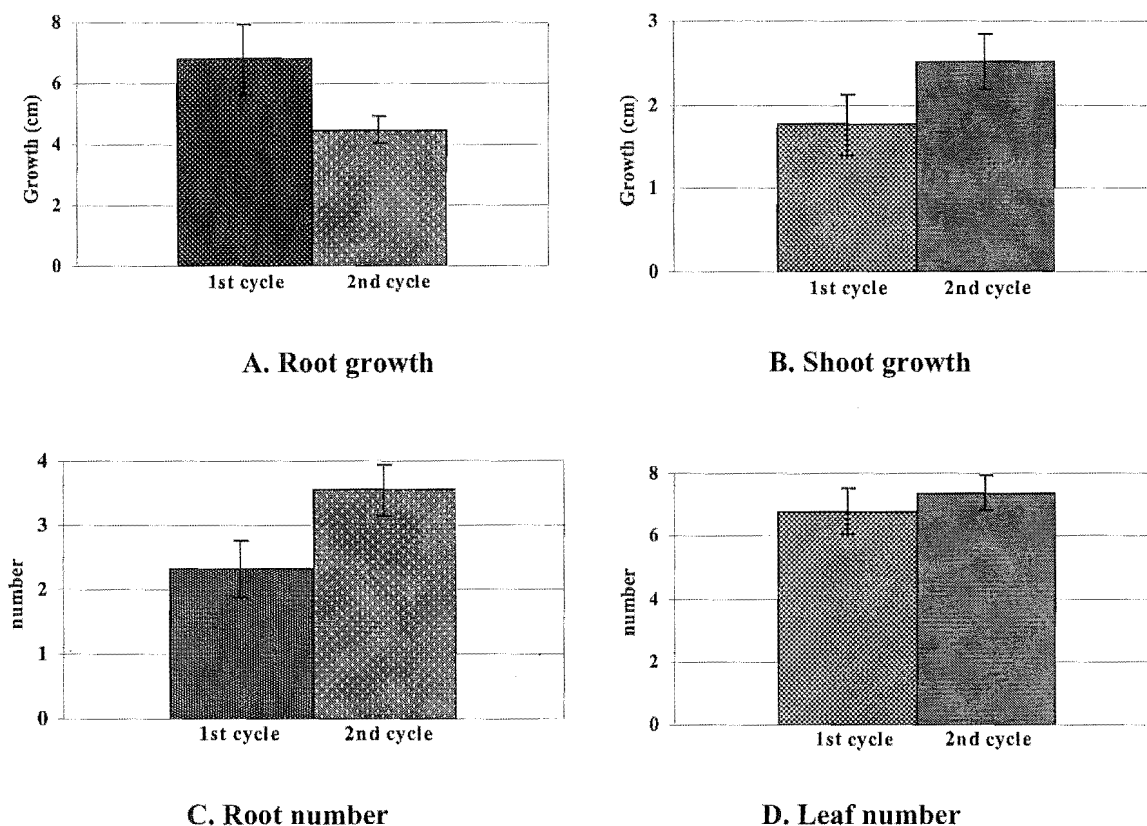


D. Leaf number

**Figure 3.5 A-D** Performance of nodal explants in successive cycles of *in vitro* propagation starting with those from *Capsicum annuum* L. cv. Sweet Banana plantlets regenerated via somatic embryogenesis. (A) root length (B) shoot length (C) number of root and (D) number of leaves. 1<sup>st</sup> cycle = first cycle of propagation; 2<sup>nd</sup> cycle = second cycle of propagation; 3<sup>rd</sup> cycle = third cycle of propagation. Duration of each cycle = 10 weeks. This experiment was repeated 3 times. In a replicate experiment, 17, 20 and 12 nodal explants were used for 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> cycle of propagation, respectively. Means  $\pm$  SE of data from one representative experiment are presented.

Statistical analysis [ANOVA,  $P < 0.05$ ; Tukey (HSD) test]

<b>A: and B:</b>	no significant difference found
<b>C:</b>	no significant difference found
<b>D:</b>	1 <sup>st</sup> cycle is significantly different from 2 <sup>nd</sup> cycle



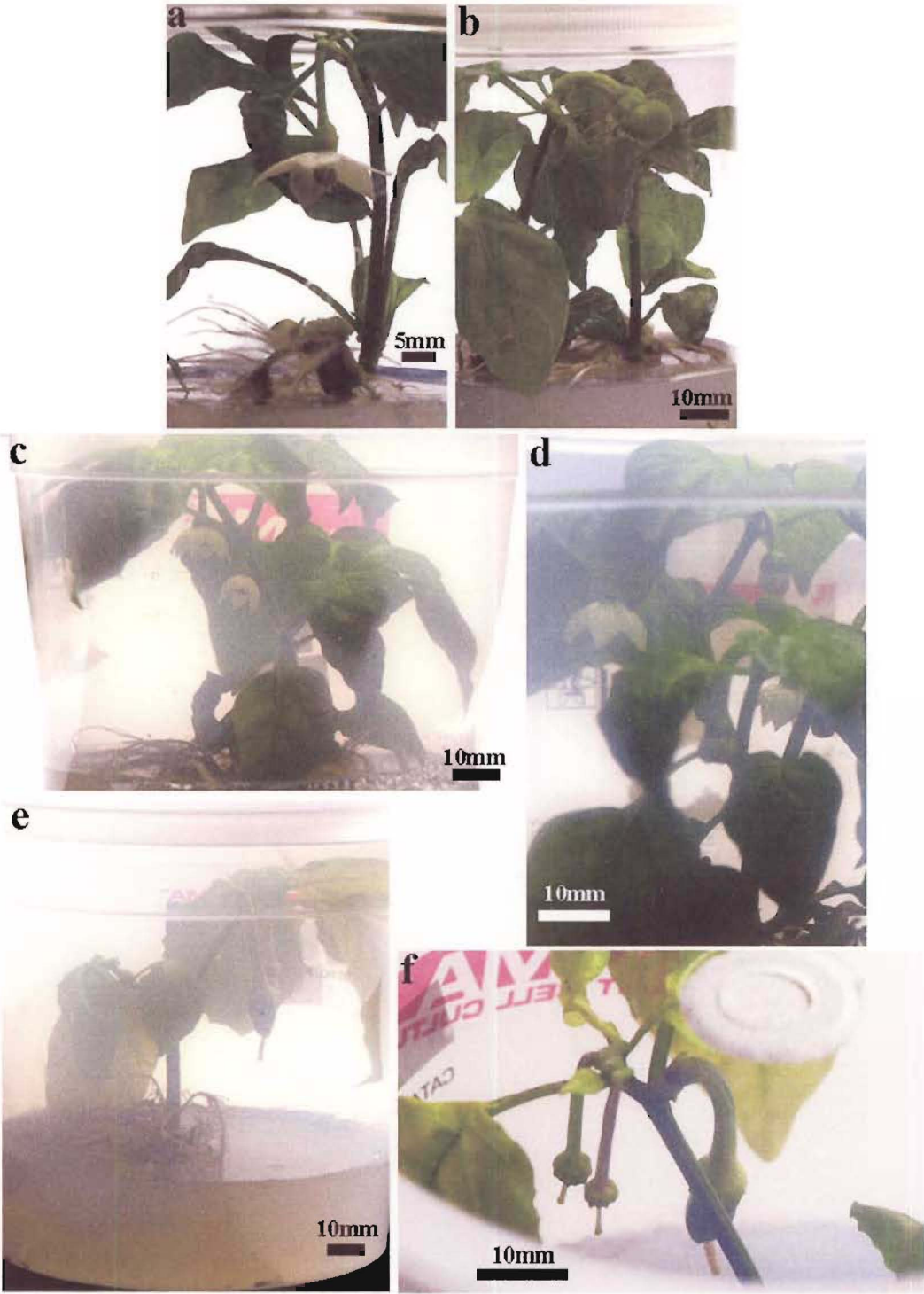
**Figure 3.6 A-D** Performance of shoot tip explants in successive cycles of *in vitro* propagation starting with those from *Capsicum annuum* L. cv. Sweet Banana plantlets regenerated via somatic embryogenesis. (A) root length (B) shoot length (C) number of root and (D) number of leaves. 1<sup>st</sup> cycle = first cycle of propagation; 2<sup>nd</sup> cycle = second cycle of propagation. Duration of each cycle = 10 weeks. This experiment was repeated 3 times. In a replicate experiment, 15 and 8 shoot tip explants were used for 1<sup>st</sup> and 2<sup>nd</sup> cycle of propagation, respectively. Means  $\pm$  SE of data from one representative experiment are presented.

Statistical analysis [ANOVA,  $P < 0.05$ ; Tukey (HSD) test]

<b>A-D:</b>	no significant difference found
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**Plate 3.19** *In vitro* flowering and fruiting on plantlets of *Capsicum annuum* L. cv. Sweet Banana grown from nodal explants. Unless specified otherwise, all the explants and regenerated plantlets were cultured on MS basal medium supplemented with 3% (w/v) sucrose in the Phytakon<sup>TM</sup> containers.

- (a) *in vitro* flowering on a plantlet after 10 weeks of culture on medium supplemented with 3% (w/v) sucrose in the small container
- (b) *in vitro* fruiting on plantlet after 12 weeks cultured on medium supplemented with 5% (w/v) sucrose in the small container
- (c) *in vitro* flowering on plantlet cultured
- (d) close up view of *in vitro* flowering on plantlet
- (e) *in vitro* fruiting on plantlet
- (f) close up view of *in vitro* fruiting on plantlet



overcome this problem, some of plantlets were transferred to the Phytacon<sup>TM</sup> containers and adding only 5% sucrose medium solution when the *in vitro* flowers were formed. After this, *in vitro* flowers and normal-looking, red ripe fruits were produced (Plate 3.19). Moreover, one of the *in vitro* fruits was found to contain 10 viable seeds which had a hard endosperm and germinated in the dark room at 26 °C and developed into normal-looking seedlings in a growth room at 23 °C with constant lighting (Plate 3.20). This result was different from those described in 3.7 section in the following respects:

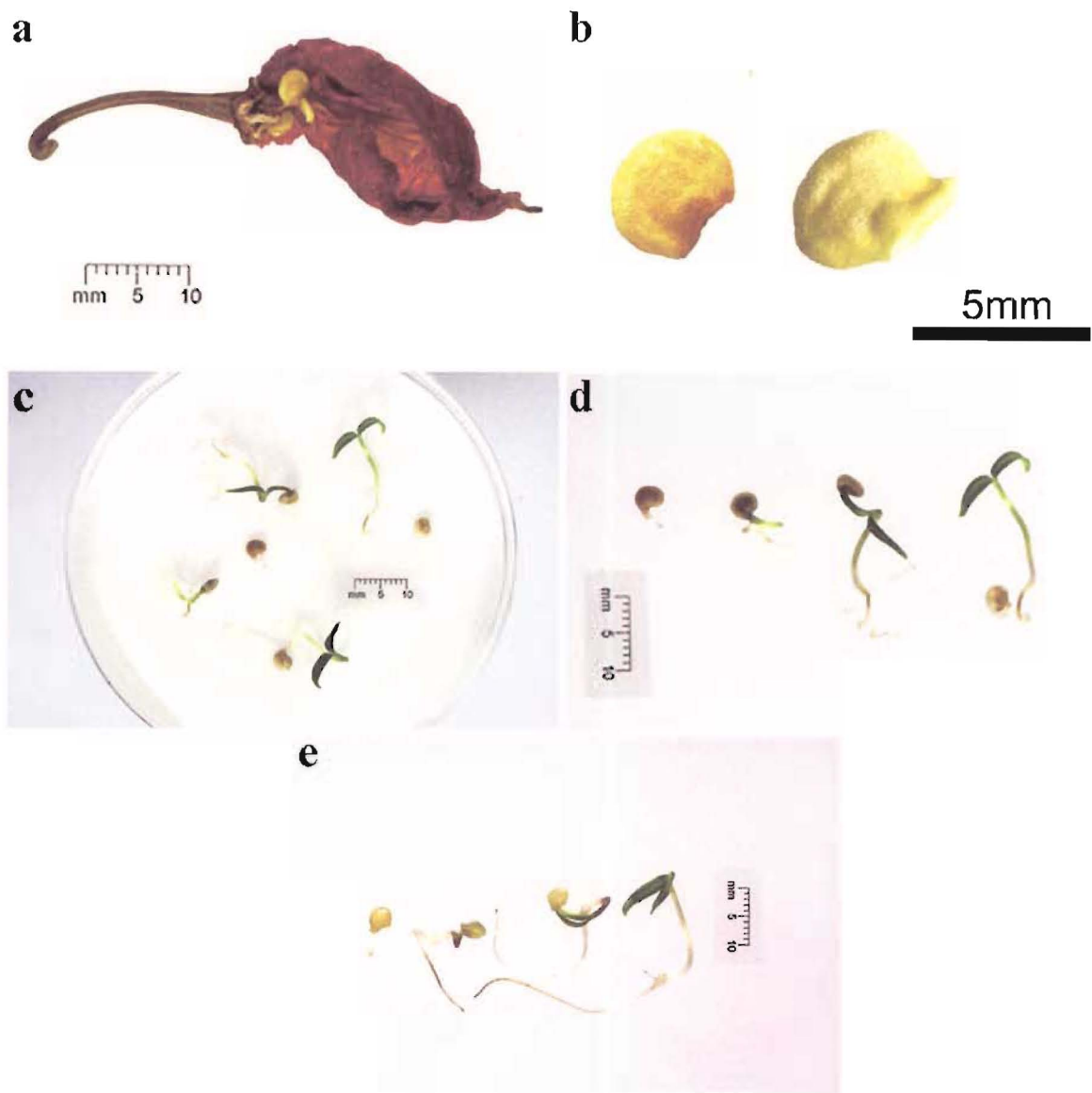
- (a) hand pollination was not needed,
- (b) silver thiosulfate solution was not added,
- (c) instead of the plantlet development medium containing 1 mg/l NAA, MS basal medium containing 3% or 5% (w/v) sucrose was used, and
- (d) probably more importantly, rather than thinning out the flowers formed to only one per plantlet in each Phytacon<sup>TM</sup> container, the plantlets were left largely unhandled during the experiment.

It remains to be determined precisely whether all or some of these differences could be critical to the production of viable and germinable seeds by the *in vitro* fruits of *C. annuum* L. cv. Sweet Banana (also see further discussion in 4.5). Nevertheless, the present results are not only interesting but also show, for the first time, that the complete life cycle starting from somatic embryogenesis of a pepper plant can take place in a container smaller than one litre.

**Plate 3.20** Germination of seeds from an *in vitro* fruit produced by an *in vitro*-propagated plantlet of *Capsicum annuum* L. cv. Sweet Banana. *In vitro* propagation was started using nodal explants from plantlets regenerated via somatic embryogenesis in immature zygotic embryo explants.

- (a) A dry *in vitro* fruit with some dry seeds (most of the seeds had been harvested while fresh and then immediately placed on moist filter paper in a Petri dish for germination test)
- (b) a close-up view of a dry seed from the *in vitro* (left) compared to one (right) from a fruit grown in the glasshouse
- (c) germination (after 12 days from sowing) of freshly harvested seeds from an *in vitro* fruit without prior air drying
- (d) a close-up view of the germinated seeds in (c)
- (e) germination (after 12 days from sowing) of dry seeds from a fruit produced by a pepper plant grown in the glasshouse





## Chapter 4

# General discussion and Conclusions

### 4.1 Major findings of this study

1. All major developmental stages of somatic embryo formation including globular, heart-shape, torpedo and cotyledonary stages had been observed during direct somatic embryogenesis in immature zygotic embryo explants from *C. annuum* L. cv. Sweet Banana.
2. On induction medium a shorter incubation time (about two weeks) than previously reported is sufficient for somatic embryo formation in immature zygotic embryo explants from four *C. annuum* L. cultivars.
3. Coconut water is not necessary for induction of somatic embryos in immature zygotic embryo explants from four *C. annuum* L. cultivars.
4. Upon further culture, the plantlets of *C. annuum* L. cv. Sweet Banana developed from germinated somatic embryos were shown to be capable of *in vitro* flowering, fruit setting, fruit ripening and seed setting in a small container (less than 1 litre capacity). This resulted from investigations aiming to overcome the problems associated with premature abscission of *in vitro* flowers and fruits, and poor seed setting.
5. The *in vitro* flowers were largely comparable to the flowers produced by the plants (cv. Sweet Banana) grown in the glasshouse.
6. Culture of nodal and shoot tip explants on plant growth regulator-free MS basal medium is a promising protocol for clonal propagation of the plantlets (cv. Sweet Banana) regenerated via somatic embryogenesis.

7. The clonal plantlets (cv. Sweet Banana) were capable of *in vitro* flowering, fruit setting, fruit ripening and seed setting when maintained for a long time (about 14 weeks) on the same medium for *in vitro* propagation. Moreover, the seeds from *in vitro* fruit could germinate and develop into normal-looking seedlings.

This is the first report showing that the whole life cycle of *Capsicum annuum* L., especially cv. Sweet Banana, could be completed starting from regeneration of plantlets via somatic embryogenesis and ending in the production of germinable seeds from the plantlets. *In vitro* flowers, fruit setting and fruit ripening have also been observed in at least two other cultivars used in this study. It is likely that the whole life cycle of most, if not all, *C. annuum* L. cultivars can be completed under *in vitro* conditions. The practical significance of this *in vitro* plant developmental capacity may not be immediately apparent at the present. However, some basic studies on various aspects of plant reproduction, fruit development, ripening and seed formation might be designed to take advantage of these processes in an aseptic environment which is more amenable to experimental manipulations.

On a biotechnological application note, *in vitro* flowers may be a good source of anthers for culture as no major difference was found when *in vitro* pepper anthers and pollen were compared with those produced by glasshouse-grown plants. Since *in vitro* anthers are produced in an aseptic environment, there is no need for surface sterilisation that might adversely affect the pollen.

It is pertinent to point out two major aspects of this research that had placed rather severe constraints on attempting many more experiments. Firstly, despite the use of glasshouse facilities, the best times of the year to initiate somatic embryogenesis experiments were in the summer and autumn months (see Appendix 4 and 5). Growth rooms and cabinets were too limited as far as the number of plants that could be grown. To compound the problem, this one-person research 'team' could only isolate a relatively small number of immature zygotic embryos for experiments on a given day. As a consequence, many fruits (and therefore seeds) at the appropriate developmental stages had to be discarded over summer months. Secondly, most, if not all, experiments in this research took weeks and months, not days, to complete. Bearing these two constraints in mind, most experiments were performed with explants of *C. annuum* L. cv. Sweet Banana.

Whenever possible, the same experiments were performed with some of the other 3 cultivars in this research.

## 4.2 Basic aspects of somatic embryogenesis

The transition from single cell growth to somatic embryo formation suggests a substantial reprogramming of gene expression that results in the development of structures that are morphologically and physiologically similar to zygotic embryos (see 1.2). *C. annuum* L. somatic embryogenesis in this research may be viewed as a useful system for investigations of the entire process of morphogenesis and differentiation of plants.

The real parent cell in the immature zygotic embryo explant that developed into a somatic embryo is still ambiguous. Had this been indirect somatic embryogenesis in a cell suspension culture, the video cell tracking technique which was applied to study somatic embryogenesis in carrot (Toonen et al., 1994) might be useful to answer this question.

Studies on the physiological, biochemical and molecular changes associated with somatic embryogenesis in pepper tissue cultures have yet to be investigated to any great extent although some biochemical changes have been studied in other systems. In particular, arabinogalactan protein, embryogenesis-related proteins, storage proteins and extracellular proteins have been shown to be associated with somatic embryogenesis in different systems.

There are huge unexplored gaps in the knowledge concerning the role of AGPs in somatic embryogenesis. Firstly, although it has been shown that mature seed extracts of carrot (Kreuger and van Holst, 1995) and Norway spruce (Egertsdotter and Von Arnold, 1995) containing AGPs could influence somatic embryogenesis in the respective cell cultures, it seems odd that mature seed could be related to the initiation of somatic embryo formation. A study of the AGPs during the development of immature zygotic embryos and evaluation of their efficacy following isolation and purification in somatic embryogenesis will shed some new light on this unanswered question.

Secondly, it would be important to establish whether AGPs from the mature seed of one species have the same effect on somatic embryo formation in another plant. Since this issue has not been addressed adequately so far, and indeed the AGPs from tissues other

than mature seeds or embryogenic cell cultures have not been evaluated for their effect on somatic embryogenesis, the experiments concerning the specificity of mature seed AGPs in somatic embryogenesis should be initiated.

Carrot and Norway spruce are not ideal systems for investigation of the above two questions. This requires a relatively convenient system under controlled conditions to study seed development but carrot is a biennial crop and Norway spruce seed development will take place outdoors subject to uncontrolled climatic variations. Thus, *C. annuum* L., which is a typical 3-4 month cycle glasshouse crop, could be a good model system chosen for this work because somatic embryogenesis has been easily obtained starting from immature zygotic embryo explants and Yarov's reagent seems to be a potent inhibitor of this process as shown in the present study. Certainly, the research examining the relationships of AGPs from various sources, different seed developmental stages and somatic embryogenesis have never been attempted in pepper or in other systems. Immunological approaches including single radial gel diffusion assay, cross electrophoresis, immunological precipitation and purification and northern blotting with AGP cDNA probes should also be used for this investigation.

Sung and Okimoto (1981) observed that carrot cell lines incapable of embryogenesis could not synthesise certain proteins. These proteins may be involved in the determination process of embryo development. The embryogenesis-related proteins were detected only after induction and remained present during embryogenesis in *Cichorium* (Hilbert et al., 1992). The present study showed that Yarov's reagent added to somatic embryo induction medium had an inhibitory effect on pepper somatic embryogenesis. Using medium with or without Yarov's reagent, would be of interest to investigate if apart from AGPs, there might be some embryogenesis-related proteins associated with pepper somatic embryogenesis.

Crouch (1982) reported that non-zygotic embryos of *Brassica napus* L. contained embryo-specific storage proteins. These were first detected in early globular-stage somatic embryos of cotton (Shoemaker et al., 1987). In the same manner, somatic embryos of Norway spruce accumulated storage proteins in abundance during their maturation phase of growth (Hakman et al., 1990). Flinn et al. (1991) also found that somatic embryos of interior spruce accumulated the same storage proteins as zygotic embryos and the storage protein levels varied among different somatic embryo genotypes; however, all genotypes

tested accumulated significant amounts of the storage proteins. In *Capsicum*, cells of the cotyledons of the immature zygotic embryo explants (Plate 3.6 c and d; 3.7 c and d; 3.8 c, d and e), apparently had some protein/lipid storage organelles. It remains to be determined if these may be associated with somatic embryo formation.

Extracellular proteins were secreted into the cell suspension culture medium by carrot (de Vries et al., 1988), barley (Nielsen and Hansen, 1992) and grapevine (Coutos-Thevenot et al., 1992) embryogenic cells during somatic embryogenesis. Satoh et al. (1986) noted that the appearance or disappearance of two glycoproteins was closely related to the formation of carrot somatic embryos. The proembryogenic masses of sour orange were also found to release glycoproteins to the medium. Addition of these glycoproteins to the culture blocked embryogenesis. These glycoproteins could play a regulatory role in *in vitro* embryogenesis (Gavish et al., 1992).

Egertsdotter et al. (1993) found that cell lines of somatic embryos with dense embryoheads secreted certain proteins to the culture medium that were essential for the development of somatic embryos of Norway spruce. Likewise, Domon et al. (1994) found extracellular proteins differed greatly between the embryogenic and non-embryogenic cell lines. Moreover, Poulsen et al. (1996) detected significant differences between the patterns of extracellular proteins secreted into the medium in which cells formed somatic embryos and in which undifferentiated cell proliferation took place.

None of the above biochemical changes reported in the literature have been investigated in somatic embryogenesis of *Capsicum annuum* L.. Thus, there is a need for future research to address this deficiency in our knowledge.

### **4.3 *Capsicum* somatic embryogenesis protocols**

At the time the present research was initiated, immature zygotic explants were by far the commonest starting materials for somatic embryo induction in many different plants including *C. annuum* L. It was thus pleasantly surprising to find the reports on somatic embryogenesis and artificial seed production starting from mature zygotic embryo explants of *C. annuum* cv. Ace (Buyukalaca and Mavituna, 1995; Buyukalaca and Mavituna, 1996). However, it had been very frustrating and wasting time (close to one year) when

despite the best attempts to follow the published protocol, no somatic embryos were obtained from mature zygotic embryo explants of cv. Ace and at least two other pepper cultivars. It should also be noted that no further reports other than those already cited here have appeared in relation to somatic embryogenesis in mature zygotic embryo explants of *C. annuum* L. Besides, convincing histological evidence for the different stages of somatic embryogenesis in mature pepper zygotic explants is lacking.

Toward the end of the present research, a new types of explant, namely young leaf, has been shown to be promising for somatic embryogenesis investigations in peppers. While the leaf-based somatic embryogenesis protocol (Kintzios et al., 1998; Kintzios et al., 2000; Kintzios et al., 2001) remains to be tested using more different *C. annuum* L. cultivars, it would be interesting to compare many findings of the present research with those using the leaf-based protocol.

Both macroscopic observations and histological work have shown that somatic embryo structures were found mainly on the cotyledons of the immature zygotic embryo explants of *C. annuum* L. An interesting question arises as to whether the cotyledons excised from the immature zygotic embryo explants would exhibit the somatic embryogenesis response. This question has apparently not been raised widely in other studies also using whole immature zygotic embryos as explants.

#### **4.4 *In vitro* fruiting**

The review on the relevant literature on *in vitro* fruiting (see 1.4) and the present result have not uncovered some complicated or unusual medium or plant growth regulator requirements for this process. It is therefore surprising that not many more reports on *in vitro* fruiting other than those few on legumes (Narasimhulu and Reddy, 1984; Dickens and Van Staden, 1985; Ignacimuthu et al., 1997; Fujioka et al., 1999; Franklin et al., 2000), brassicas (Kumar et al., 1995), strawberry (Asao et al., 1997), tomato (Gamborg, 1995) and peppers (Tisserat and Galletta, 1995; this thesis).

From these few studies and the present results, it seems that the physical culture environment is one of the important keys to the initiation and sustained development of *in vitro* fruits. This is not dissimilar from the finding of the regulation of premature fruit

abscission by silver thiosulfate in the glasshouse-grown plants of *C. annuum* L. cv. Maor (Aloni et al., 1995). In a small tissue culture container as used in this research, the gaseous environment (probably involving ethylene) was found to be particularly challenging for successful *in vitro* fruit formation and ripening. A study of ethylene production and action in relation to *in vitro* fruiting seems worthwhile.

#### 4.5 Apparent key to the success of *in vitro* seed production

Early on in this research it was shown that hand pollination was beneficial for *in vitro* fruiting but was not good enough for producing germinable seeds. From the clonal propagation experiments, a possible reason has emerged. In earlier experiments, most of the *in vitro* flowers produced by a plantlet were removed to collect pollen for hand pollination trials. In contrast, the *in vitro* fruits that produced germinable seeds without prior hand pollination were from the clonal plants that each had at least eleven *in vitro* flowers. The flowers were not removed from the plant. Moreover, before fruit setting, there were four to five flowers blooming on the plant at the same time. Thus, the production of germinable seeds *in vitro* may require a sufficient supply of good quality pollen provided in favourable self-pollination conditions. Further research is needed to conduct a large scale experiment to compare the effect of hand pollination and self pollination on *in vitro* pepper seed production.

#### 4.6 Future research

Developmental biology of plants is a vast field and has many intriguing problems. Plant tissue culture is a valuable tool in this field. Many avenues for further research on the research problems in this thesis have already been discussed. In particular, arising from the present study, there are many points of immediate interests that could be investigated further. They are listed below:

1. The effect of maltose and other sugars on somatic embryogenesis in different *Capsicum* cultivars.



2. A closer examination of the possible role of AGPs in *Capsicum* somatic embryogenesis.
3. The potential of *in vitro* propagation of *Capsicum* plantlets beyond three successive cycles.
4. Application of the clonal propagation protocol to seedlings from natural seeds with the aim of ascertaining if germinable seeds could also be produced via *in vitro* fruiting of the clonal plantlets.
5. Experimentation to see if the present research findings being on the relatively large-fruited *Capsicum* cultivars could also be applied to produce *in vitro* fruits in small-fruited *Capsicum* species, for example, hot chillies.
6. The question whether somatic embryos induced in leaves of pepper plants (Kintzios et al., 2000; Kintzios et al., 2001) could complete the life cycle as described in this research.
7. The factors influencing pepper somatic embryo germination or plantlet conversion; these events may have requirements different from those associated with somatic embryo induction.
8. A more thorough comparative study on the effect of hand pollination and self pollination on *in vitro* seed production, with special emphasis on the number of germinable seeds produced.
9. With the knowledge of *in vitro* flowering, fruiting and seed production in the present study and using the protocol for pepper androgenesis (Dolcet-Sanjuan et al., 1997), a study of the whole life cycle of *C. annuum* L. starting from regeneration of plantlets via androgenesis using *in vitro* pollen and ending in the production of germinable seeds from the androgenetic plantlets.
10. An examination of the capacity of immature zygotic embryo explants from *in vitro* seeds for somatic embryogenesis. A year-round supply of fresh immature zygotic embryo explants for this type of work might be achieved without dependence on the rather limited and unreliable supply of seeds from glasshouse-growth plants.
11. Use of transmission electron microscope (TEM) to investigate ultrastructural changes associated with somatic embryo formation.

12. Comparison of the *in vitro* and *in vivo* flowers (including pollen morphology and physiology), fruits and seeds in other pepper cultivars to ascertain the finding in this present research.
13. The potential of cryopreservation technique to preserve immature zygotic embryo explants as a means to ensure supply of material for somatic embryogenesis when required.
14. Transformation of immature zygotic embryo explants from *C. annuum* L. cv. Sweet Banana taking advantage of the findings about the factors important for somatic embryogenesis in this type of explants.

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# Appendix 1

## Preparation of media

### 1. Murashige & Skoog (1962) stock solutions

#### Major Salts Stock Solution (10 X)

$\text{KH}_4\text{NO}_3$	16.5 g
$\text{KNO}_3$	19.0 g
$\text{CaCl}_2 \cdot \text{H}_2\text{O}$	4.4 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	3.7 g
$\text{KH}_2\text{PO}_4$	1.7 g
dH <sub>2</sub> O-bring volume to	1 L

Store at 4°C.

#### Minor Salts Stock Solution (100 X)

KI	0.083 g
$\text{H}_3\text{BO}_3$	0.620 g
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	2.230 g
$\text{ZnSO}_4 \cdot 5\text{H}_2\text{O}$	0.860 g
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.0025 g
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.0025 g
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.0250 g
dH <sub>2</sub> O-bring volume to	1 L

Store at 4°C.

Organic Supplements Stock Solution (100 X)

Myo-inositol	5000 mg
Nicotinic acid	25 mg
Pyridoxine-HCl	25 mg
Thiamine-HCl	25 mg
Glycine	100 mg
dH <sub>2</sub> O-bring volume to	500 ml

Store at 4°C.

Iron (Fe-EDTA) Stock Solution (50 X)

Solution A: FeSO<sub>4</sub>·7H<sub>2</sub>O                      1.390 g in 200 ml dH<sub>2</sub>O

Solution B: Na<sub>2</sub>EDTA·2H<sub>2</sub>O                      1.865 g in 200 ml dH<sub>2</sub>O

Solutions A and B were mixed, the volume made up to 500 ml and stored at 4°C in a dark bottle.

**2. Plant Growth Regulators Stock Solutions**2,4-D, GA3, NAA and PAA Stock Solutions

Stock solutions (1 mg/ml) of each of the above plant growth regulators were prepared by first dissolving in a small amount of 70% (v/v) ethanol before making up to the required volume with dH<sub>2</sub>O.

BA Stock Solution

A stock solution (1 mg/ml) of was prepared by first dissolving in a small amount of 1M NaOH before making up to the required volume with dH<sub>2</sub>O.

All plant growth regulator stock solutions were stored at 4°C.

### 3. Other Stock Solutions

#### AgNO<sub>3</sub> Stock Solution

AgNO <sub>3</sub>	0.0169 g
dH <sub>2</sub> O-bring volume to	10 ml

#### Ag<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (STS) Stock Solution

1. AgNO <sub>3</sub>	0.0102 g
dH <sub>2</sub> O-bring volume to	5 ml
2. Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> ·5H <sub>2</sub> O	0.1191 g
dH <sub>2</sub> O-bring volume to	5 ml

Equal volumes of 1 and 2 were mixed.

## **Appendix 2**

# **Glasshouse work**

Plants of the four cultivars of *Capsicum annuum* L. used in this research were grown separately in the glasshouses at the University of Canterbury glasshouse. Immature seeds at the appropriate developmental stages were obtained from these plants for experiments over a period of 3 years.

### **1. Seed germination, seedling and plant manipulation**

Pepper seeds were immersed in distilled water overnight. After this, seeds (15 in a 10 cm X 15 cm container) were sowed in a potting mix with a slow release fertiliser (see later). The container were covered with a sheet of plastic and kept in a growth room (25 °C and 16 hours photoperiod). The seedlings began germinating at around 9-10 days from sowing. Fifteen days later, the seedlings were transferred to the glasshouses.

One month later, pepper plants were transplanted to larger pots (15 cm high X 14-17 cm diameter) with newly added fertiliser and were placed under the shade. At this stage, the pots were placed close together. After the plants were strong enough, the space between pots was increased to 45-60 cm. The flower buds began to appear at around 1-1.5 months after transfer of the seedlings to the larger pots. Each flower was labelled with a writable paper tag (see later) as it began opening (anthesis). Some fruits were selected at various stages as a source of immature embryos for somatic embryo induction experiments.

## **2. Glasshouse condition**

There was a central heating system to keep the glasshouse temperatures at between 21-28°C. On the ceiling of the glasshouses, there was a retractable shade screen or curtain to modulate the intensity of the incoming sunlight. In addition, there were 2 vents in the glasshouse ceiling that can open and close automatically as a part of temperature control inside the glasshouse. However, in the summer months (December to February) the temperature inside was sometimes over 35 °C. The plants were watered using an automatic control system twice a day (9 am and 3 pm). In each glasshouse (3 m X 3 m), 30 pots (each with a capacity of 2.5 litres; 15 cm high X 14-17 cm diameter) could be placed with 50 cm space between each pot.

## **3. Soil material**

The potting mix used contained a slow release fertiliser lasting for 8-9 months. Its composition was 60% bark, 20% peat, 10% sand, 10% (w/v) sterilised soil with Nutricote® 8-9 months slow release (N: P: K: Mg = 18: 2.6: 6.6: 1.2 + trace elements), or the slow release Nitrophoska® blue extra 12-5-14-6 (BASF) lasting for 3-4 months.

## **4. Caring of plants**

Many insects, mainly, aphids, caterpillar, white flies, spider mites and slugs had been found throughout the year in the glasshouse. Insecticide spray was required from time to time.

The wet wool mat placed under the pots was used to supply water to the plants for a longer period of time but it was also a good place for slugs and snails. If sand or tiny stones were used to cover the surface of soil, it seemed to have reduced the air ventilation inside the pot. Many roots grew up over the sand as a result. Thus the soil surface in the pots were not covered with sand.

Using retractable shade screen or curtain was useful to prevent sunburn damage of the young fruits (less than 30 days old), especially in cvs. California Wonder, Yolo Wonder and Ace, from direct sunlight in the summer months.

In winter, artificial light (16 hours daily) had been supplemented in the glasshouses. Plants were found growing well with big green leaves, a lot of lateral buds and big fruits but seeds were seldom found inside the fruits.

## **5. Labelling**

Since fruits of a suitable or certain developmental stage were required for experiments, a good tag identification system was designed. Strung tags (Globe, New Zealand) were used to record the date of opening (anthesis) of each flower produced by a plant. The tags were hung on the flower stalks.

Typically, the flowers of 4-5 plants of each cultivar were selected randomly for labelling. On a given day, the anthesis of one flower produced by each of the selected plants was labelled. On each plant, only 5-6 normal fruits in cv. Sweet Banana and 3-4 normal fruits in cvs. California Wonder, Yolo Wonder and Ace were maintained. Excess flowers were removed (thinning) to get high quality fruits. Abnormal flowers and fruits were also discarded.

Generally, since the first flower opening, other flowers continued anthesis until 20-30 days. When the first fruit developed further, the flowers on the same plant began dropping on their own. Keeping only one fruit on each branch led to the development of a good quality fruit in terms of seed size and ability of immature zygotic embryo for somatic embryogenesis.

## Appendix 3

### Some horticultural characteristics

According to Hartmann et al. (1981), some characteristics of some of the cultivars used in this research which might be of interests are listed in the following table.

Characteristic	Sweet Banana	California Wonder	Yolo Wonder
Days to maturity	72	75	75
Size (cm)	14 x 4	10 x 10	10 x 9
Fruit bearing	Pendant	Upright	Pendant
Fruit walls	Thin	Thick	Thick
Flavour	Sweet	Sweet	Sweet
Remarks	Good for frying, ornamental	Standard bell type	Blocky fruit

Note: no information found about the cv. Ace.

## Appendix 4

### Seasonal effect I

Influence of seasons on the availability of immature zygotic embryo explants of *Capsicum annum* L. cv. Sweet Banana for somatic embryogenesis experiments.

Season	Fruit age (days)*	Number of seeds per fruit*	Number of appropriate embryos explants per fruit*
Summer	42.5±0.4	62.9±5.9	14.2±0.6
Autumn	47.3±1.3	92.7±9.9	11.4±0.8
Winter	53.5±0.9	52.4±12.8	15.4±3.4
Spring	45.4±0.4	125.5±15.8	15.3±1.9

\*Values shown are means±S.E. of data obtained from 46, 30, 5 and 8 replicate experiments in the case of Summer, Autumn, Winter and Spring respectively. Age of fruits containing seeds with immature zygotic embryo explants that were judged to be as appropriate explants for somatic embryogenesis (see 3.2.1)

Note: A lot of fruits had no seed inside during Winter and Spring



## Appendix 5

### Seasonal effect II

Influence of seasons on somatic embryogenesis of *Capsicum annuum* L. cv. Sweet Banana.

Season	Number of explants	% Induction*	% Conversion*	SE number*	% Healthy plants*
Summer	654	68.3±2.8a	60.6±2.8a	2.7±0.1a	8.3±0.5a
Autumn	342	60.5±4.2a	52.9±4.1a	2.1±0.1ab	3.7±0.2a
Winter	77	27.8±11.9b	23.6±10.2b	1.1±0.5b	0
Spring	122	49.7±15.4ab	41.5±13.1ab	2.0±0.4ab	9.4±1.1a

\*Values shown are means±S.E. of data obtained from 46, 30, 5 and 8 replicate experiments using explants in Summer, Autumn, Winter and Spring respectively. Data from a cultivar marked by same letter in a column are not significantly different (ANOVA,  $P < 0.05$ ). Number of explants = immature zygotic embryos used in all the replicate experiments; % induction = the percentage of immature zygotic embryo explants that formed somatic embryo structures; % conversion = the percentage of immature zygotic embryo explants that formed plantlets (whether they appeared normal or not); SE number = number of somatic embryo structures forming per explant; % Healthy plants = the percentage of plantlets which appeared normal.

## Appendix 6

# Plant Microtechniques

(R. Gardiner, personal communication)

### 1. Phosphate buffer (0.075 M Phosphate buffer, pH 7.2)

Prepare buffer A and B stock solutions

Buffer A stock solution =  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  (17.805 g/500 ml)

Buffer B stock solution =  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  (15.605 g/500 ml)

Both stock solutions should be kept at room temperature, as they will precipitate out at 4°C.

Mix the following:

Buffer A = 36 ml

Buffer B = 14 ml

distilled water = 82 ml

This mixture can be kept at 4 °C for about one week.

### 2. Glutaraldehyde fixative

Mix 2-3 ml of glutaraldehyde (50% or 25%) with 97 ml of phosphate buffer prepared in 1.

Caution: Gloves and glasses were worn in preparing and handling solutions described in 2-6 here.

### **3. Liquid resin**

Prepare a fresh solution just before use as follows.

Dissolve catalyst (hardener I) in liquid resin (0.1 g catalyst per 10 ml resin). Caution: don't let the catalyst dry as it can explode when dried) Add liquid resin into the beaker, using a magnetic bar to stir until the catalyst is dissolved.

### **4. Embedding resin**

Prepare a fresh solution just before use as follows.

Infiltration solution: same as liquid resin in 3.

(can make up to 40 sample blocks from a 10 ml mixture)

Add hardener II slowly into the infiltration solution while stirring gently (1 ml of hardener II per 15 ml of infiltration solution). Avoid creating any air bubbles in the mixture.

This mixture had to be used within 4-10 minutes after its preparation.

### **5. Support resin**

Prepare support resin as per Technovit® 3040 kit instructions [use 2 parts powder to 1 part liquid (v/v)]. Polymerisation of this resin occurs in 2 minutes.

### **6. Stain solutions**

1% methylene blue:

Dissolve 0.1 g methylene blue in 10 ml distilled water.

1% Azure II:

Dissolve 0.1 g Azure II in 10 ml distilled water.

Equal volumes of methylene blue and Azure II solution were mixed.

This mixture was used to stain the histological sections.

## Appendix 7

# Pollen viability and germination tests

### 1. Pollen viability test

Alexander's stain (Alexander 1969) was used for determining viability of pollen from both *in vitro* and *in vivo* pepper flowers. The Alexander's stain consists of the following chemicals:

95% (v/v) ethanol	10 ml
malachite green	1 ml [1% (w/v) malachite green in 95% (v/v) ethanol]
distilled water	50 ml
glycerol	25 ml
phenol	5 g
chloral hydrate	5 g
acid fuchsin	5 ml [1% (w/v) acid fuchsin in water]
orange G	0.5 ml [1% (w/v) orange G in water]
glacial acetic acid	1-2 ml

## 2. Culture medium (Mercado et al., 1994) for pollen germination test

Boric acid (0.1 mM) 0.0012 g

Calcium chloride di-hydrate (1 mM) 0.0294 g

Sucrose (5%; w/v) 10 g

Agar (0.8%; w/v) 4 g

The medium (200 ml) was prepared by mixing the above substances with distilled water, autoclaved at 121 °C and 15 *psi* for 20 minutes, and then poured into Petri dishes (80 mm diameter).

## Appendix 8

### *In vitro* propagation using lateral shoots

#### **Clonal propagation using lateral shoots from decapitated *C. annuum* L. cv. Sweet Banana seedlings**

Preliminary experiments showed that explants from the lateral shoots of detipped plantlets of *C. annuum* L. cv. Sweet Banana that were previously established from shoot tips of somatic embryo-derived plantlets grew poorly on the MS basal medium comprising 3% (w/v) sucrose. This was also confirmed in the following experiment.

Seedlings were raised on sucrose-free MS basal medium for 14 days (Plate A1 a), from the seeds produced by somatic embryo-derived plants grown in the glasshouse. Shoot tips of these seedlings were excised (Plate A1 b) and placed on MS basal medium comprising 3% (w/v) sucrose. Rooting occurred in these explants after 10-12 days and a growing plantlet resulted with shoot development occurring within 4 weeks of culture (Plate A1 c). After the shoot of the plantlet had developed 4-5 nodes (Plate A1 d and e), the shoot tip was removed (Plate A1 f) to allow the new 1<sup>st</sup> lateral shoot to develop from the remaining part of the original shoot (Plate A1 g).

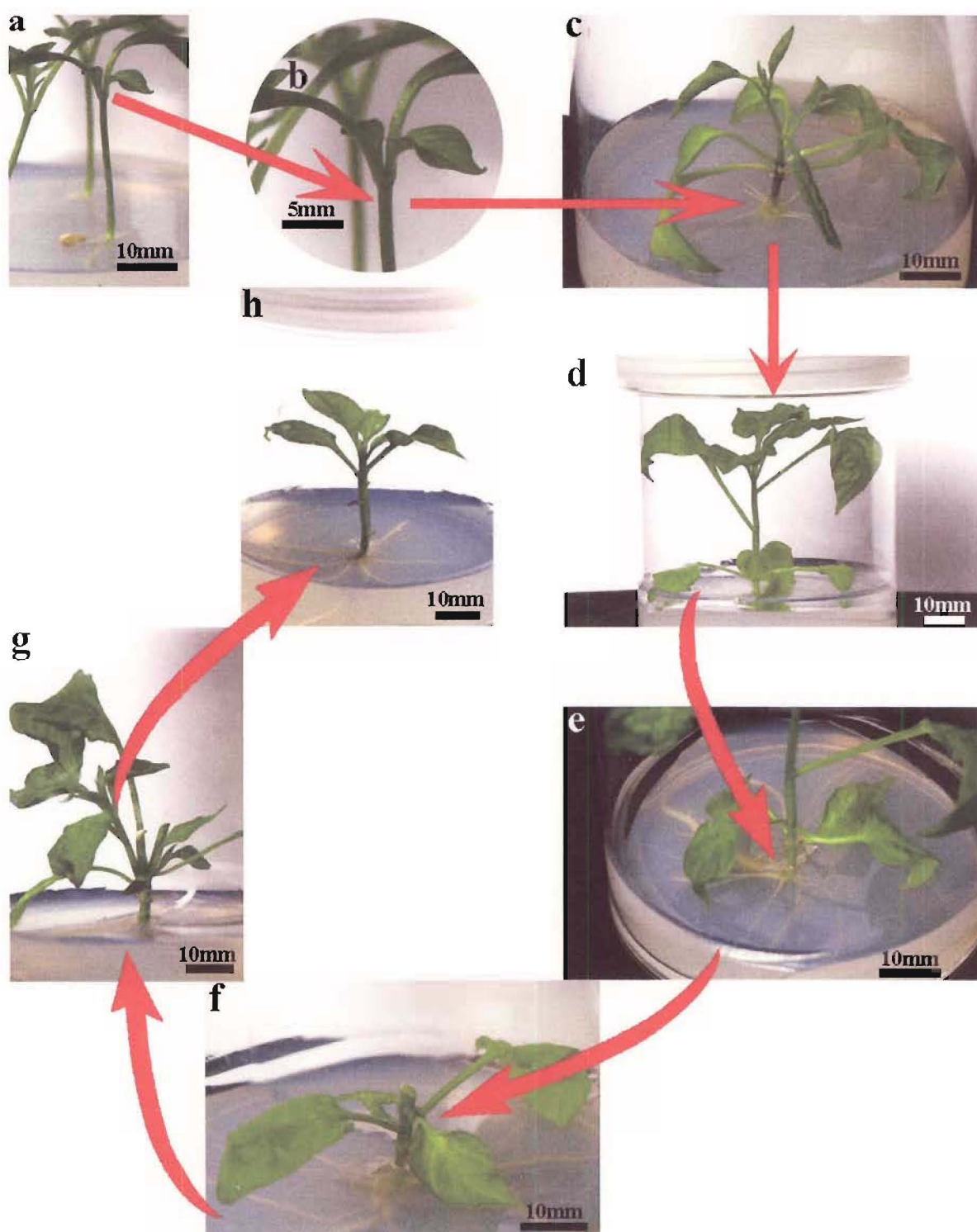
These lateral shoots developed to 4-5 nodes before nodal segments and shoot tips were excised from them and placed on MS basal medium comprising 3% (w/v) sucrose. After this, the decapitated plantlets were still left on the original medium to allow 2<sup>nd</sup> lateral shoots to emerge in order to obtain nodal and shoot tip explants of these lateral shoots to see if they can regenerate plantlets. However, it was observed that decapitated explants were not able to initiate 2<sup>nd</sup> round of lateral shoot formation and some of the lateral shoots formed appeared very weak and slow growing. It was also found that excised shoot tip explants from the 1<sup>st</sup> lateral shoots (Plate A1 h) seem to develop slightly better

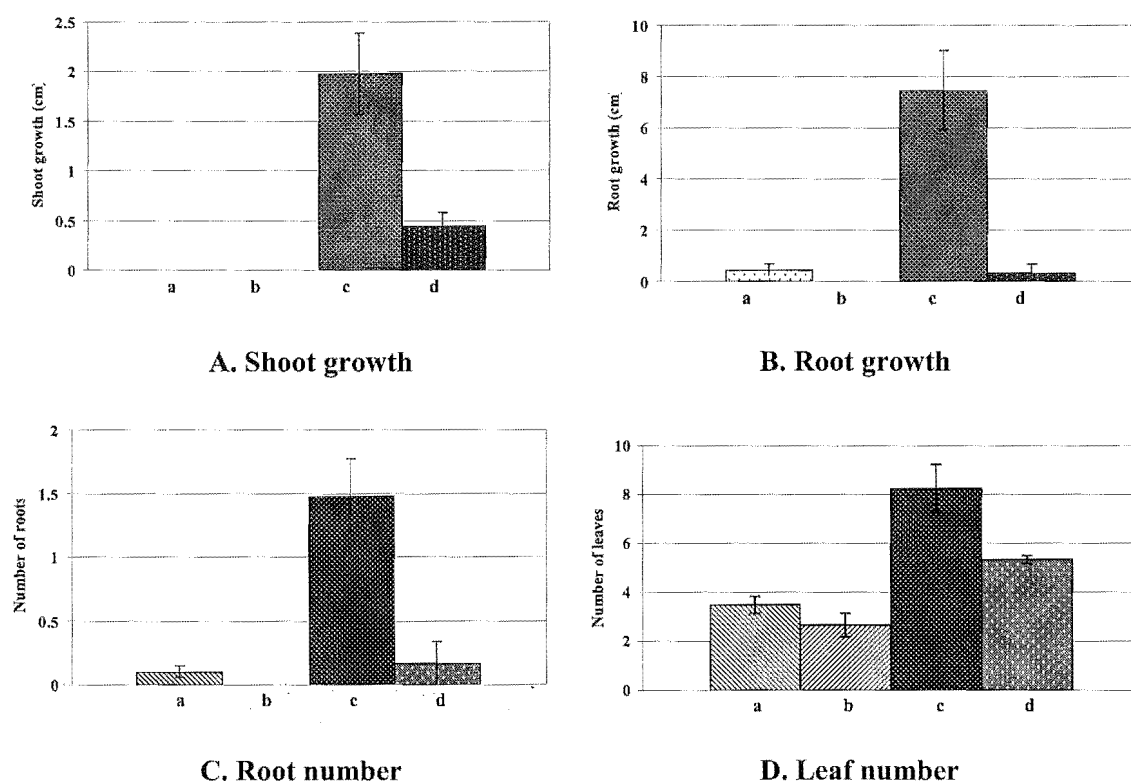
than those of the 2<sup>nd</sup> lateral shoots (Figure A1 A-D). Perhaps, the only exception was that root growth (length) was obviously more in the shoot tip explants from the 1<sup>st</sup> round of lateral shoots than those of the 2<sup>nd</sup> round. Moreover, in general nodal explants from both rounds of lateral shoots showed inferior development to the shoot tip explants (Figure A1: A-D).

**Plate A1** Clonal propagation starting with shoot tip explants of seedlings raised from seeds produced by somatic embryo-derived plants of *Capsicum annuum* L. cv. Sweet Banana grown in the glasshouse. Seedlings and all explants were grown on MS basal medium containing 3% sucrose.

- (a) 14 days old seedlings
- (b) excision to obtain shoot tip explant as indicated by the arrow
- (c) development of a shoot tip after 4 weeks of culture
- (d) development of a plantlet with 4-5 nodes from the shoot tip explant after 6 weeks of culture
- (e) a close-up view of the plantlet in (d) showing its well-developed roots
- (f) the plantlet in (d) was cut to remove the shoot tip and 3 nodes below so that the remaining part of the plantlet (decapitated plantlet) was growing
- (g) 1<sup>st</sup> lateral shoot developed from the decapitated plantlet
- (h) development of a shoot tip explant from the 1<sup>st</sup> lateral shoot after 4 weeks of culture







**Figure A1: A-D** Comparison of development of nodal and shoot tip explants from the 1<sup>st</sup> and 2<sup>nd</sup> rounds of lateral shoots produced by the decapitated seedlings of *Capsicum annuum* L. cv. Sweet Banana. (A) shoot length, (B) root length, (C) number of roots and (D) number of leaves. a and b = nodal explants from the 1<sup>st</sup> and 2<sup>nd</sup> rounds of lateral shoots, respectively; c and d = shoot tip explants from the 1<sup>st</sup> and 2<sup>nd</sup> rounds of lateral shoots, respectively. This experiment was repeated 3 times. In a replicate experiment, 17 and 7 nodal and 7 and 6 shoot tip explants were used for the 1<sup>st</sup> and 2<sup>nd</sup> round of lateral shoots, respectively. Means  $\pm$  SE of data from a replicate experiment are presented.

Statistical analysis [ANOVA,  $P < 0.05$ ; Tukey (HSD) test]

<b>A:</b>	a,b and d are significantly different from c
<b>B:</b>	a,b and d are significantly different from c
<b>C:</b>	a,b and d are significantly different from c
<b>D:</b>	a and b are significantly different from c